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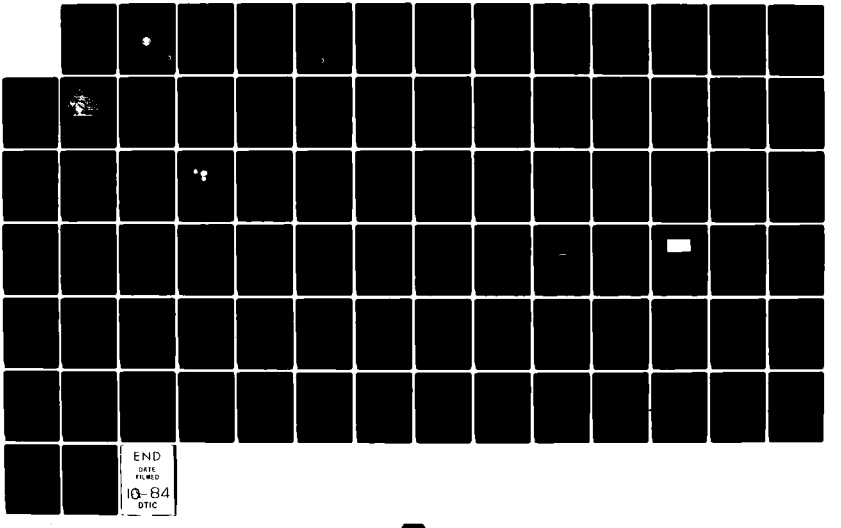

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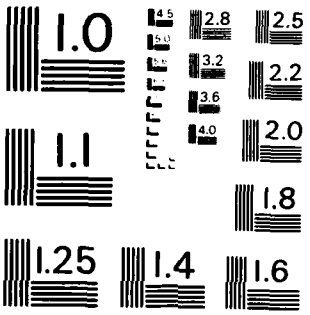

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A TRIDENT SCHOLAR PROJECT REPORT

NO. 129

THE EFFECT OF CONTROLLED PRESSURE CHANGES ON THE STIMULATION OF BIOLUMINESCENCE IN PYROCYSTIS LUNULA

UNITED STATES NAVAL ACADEMY
ANNAPOLIS, MARYLAND

1984

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Bioluminescence in a dinoflagellate species, Pyrocystis lunula, was stimulated by controlled, repeated pressure changes. Pressure pulses of a two-second duration were used to determine their effect on stimulated bioluminescence. Observations of organism sensitivity in response to the circadian rhythm, light phase, and cell fatigue were also made. The pressure change was effected by valve-regulated compressed air. The luminescence was detected with a photomultiplier tube. The mean threshold for luminescence in

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Pyrocystis lunula was found to be 5.10±1.70 psi. Pressure decreases were found to be much more effective than pressure increases. Three other rates of pressure change were investigated and a rough correlation was established between higher rates of pressure change and slightly lower threshold levels. Qualitative observations indicated that increased rates of pressure change were also associated with higher initial flashes and faster fatigue times. Pulse length appeared not to affect the stimulable luminescence to any significant degree. Measurements made at various times in the scotophase revealed a relatively constant, high level of light output, while only minimal, if any, light output was detected in the photophase.
The resulting threshold level was applied to a pressure field model around a submerged cylinder and the probable location of bioluminescence was predicted.

"THE EFFECT OF CONTROLLED PRESSURE CHANGES ON THE STIMULATION OF BIOLUMINESCENCE IN PYROCYSTIS LUNULA"

A Trident Scholar Project Report

by

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ABSTRACT

Bioluminescence in a dinoflagellate species, Pyrocystis lunula, was stimulated by controlled, repeated pressure changes. Pressure pulses of a two-second duration were used to determine their effect on stimulated bioluminescence. Observations of organism sensitivity in response to the circadian rhythm, light phase, and cell fatigue were also made. The pressure change was effected by valve-regulated compressed air. The luminescence was detected with a photomultiplier tube. The mean threshold for luminescence in Pyrocystis lunula was found to be 5.10 ± 1.70 psi. Pressure decreases were found to be much more effective than pressure increases. Three other rates of pressure change were investigated and a rough correlation was established between higher rates of pressure change and slightly lower threshold levels. Qualitative observations indicated that increased rates of pressure change were also associated with higher initial flashes and faster fatigue times. Pulse length appeared not to affect the stimulable luminescence to any significant degree. Measurements made at various times in the scotophase revealed a relatively constant, high level of light output, while only minimal, if any, light output was detected in the photophase. The resulting threshold level was applied to a pressure field model around a submerged cylinder and the probable location of bioluminescence was predicted.

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I. INTRODUCTION

Recently, marine bioluminescence has come under increased scrutiny as the result of its potential impact on optical detection and communication systems. In order to evaluate the influence of bioluminescence on these systems, a much more complete understanding of the luminescence process is required. This project investigated one aspect of the stimulation required to cause light emission. The basic objective was to make quantitative measurements of the stimulation necessary to trigger a bioluminescent response. There were four specific experimental objectives of this project. The first was the determination of the minimum level of stimulation necessary to induce light emission. The second was an analysis of the effect of varying rates of pressure change on the threshold level. Observations of light characteristics and the influence of other variables, such as the circadian rhythm, formed the third objective. The final objective was to apply the generated threshold levels to a streamline flow model, and determine where bioluminescence would occur in relation to the hull form.

A. Hypothesis

The results of previous experiments, especially those conducted by Donaldson (1980), helped in the development of a general hypothesis for the experiment. Controlled pressure changes of relatively small magnitudes, less than half an atmosphere, would induce stimulation of bioluminescence.

Decreases in pressure were expected to be considerably more effective than increases. A potential correlation between higher rates of pressure change and lower threshold levels was also investigated. The experiment tested these hypotheses and those results, accompanied by observations of other factors influencing light emission and a background on the phenomenon of bioluminescence are presented in this paper.

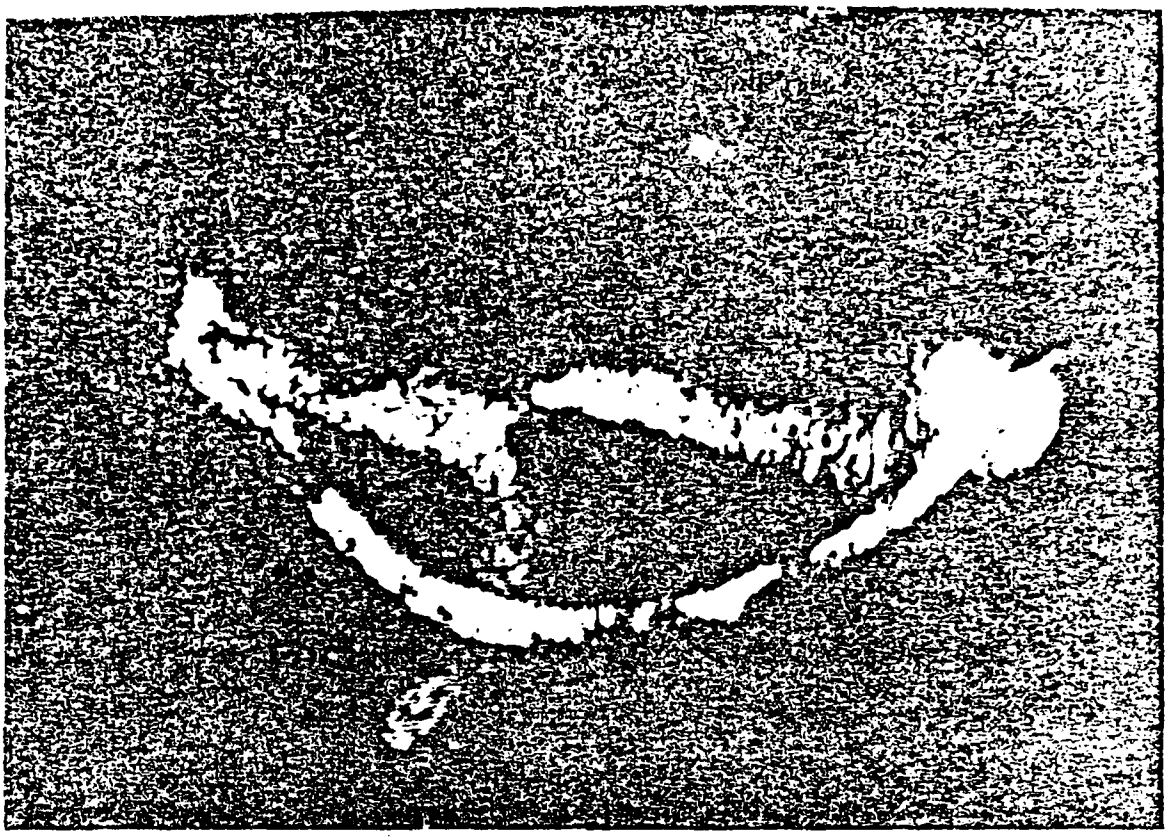
B. Importance to the Navy

The influence of bioluminescence in the Navy is felt primarily in detection and communication. The movement of surface ships and submarines through concentrations of luminescent organisms allows optical detection of these vessels. This is an additional tool that, when used in conjunction with acoustic and electronic sensors, could enhance antisubmarine warfare tactics significantly.

Several studies have investigated the relationship of bioluminescence and antisubmarine warfare (Carlson, 1978; Knight, 1980). The Soviet Navy has expressed considerable interest in this area, as demonstrated by their many publications on the subject. Through the interest expressed in the Soviet scientific literature, as well as indications of much current research in bioluminescence, it was evident that the Soviets are pursuing this field actively (Stiffey, 1983).

The commercial fishing industry has realized the potential use of bioluminescence as an aid to locate schools of fish. A feasible bioluminescence detection system would have to overcome two basic limitations. The first would be the

frequent occurrence of very dim bioluminescent displays that are well below the threshold visible to the human eye. This would require photomultiplier radiometers that are very sensitive to small amounts of light. Currently available low light sensors, also known as low light level image intensifiers (LLLII), amplify light up to 130,000 times (Lynch, 1982). Obtaining adequate observations of bioluminescence would pose the second major limitation to optical detectors, since bioluminescence is widely scattered in every ocean of the world. Ship reports are insufficient by themselves because of the uncertainty introduced as the result of variations in shipping density. With the intent of resolving this problem, several experiments have been conducted using LLLII's aboard aircraft to survey large areas (Athey, 1979 and Lynch, 1982). The use of these sensors has proved quite successful, especially as employed by the National Marine Fisheries Service. Fish schools have been located, and even particular species have been identified from altitudes of up to 6000 feet (Lynch, 1982). An example of this is shown in Figure 1 (Blaxter and Hunter, 1982). In addition, detailed images of fish trawls submerged to depths of 30 to 90 feet have been obtained from a LLLII at an altitude of 1500 feet (Lynch, 1982). The accuracy of these images indicates that an optical detection system based on luminescence would have feasible naval applications. Further refinement of the sensors would lead to deployment on satellites, allowing global coverage at regular intervals.



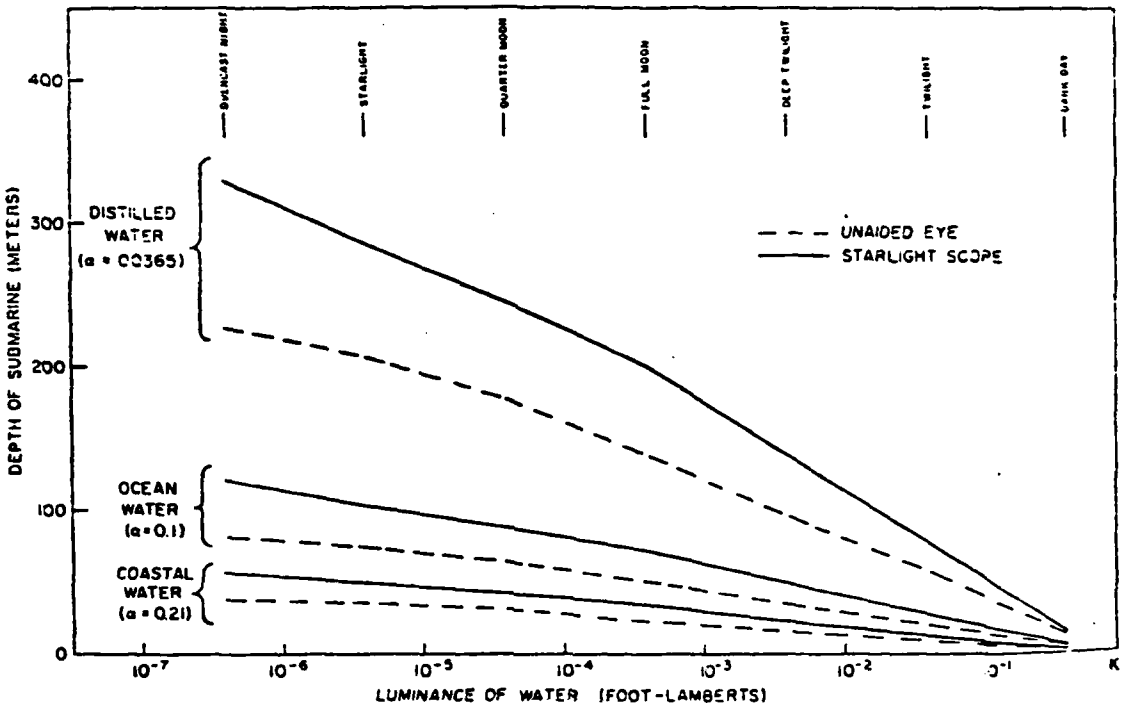
Aerial photograph of anchovy fishing from 6000 feet taken at night using image intensification. The downwardly curved bow is the net being set by the seine skiff which is on the far left. The circle of light marking the skiff is its navigation light. On the far right is the mother ship again marked by its navigation lights. Both the net and anchovy school which is T-shaped are marked by bioluminescence. Part of the school is escaping below the net and another part, far right has moved below the mother ship (unpublished photograph by permission of J. Squire, Southwest Fisheries Center, La Jolla).

FIGURE 1: IMAGE-INTENSIFIED AERIAL PHOTOGRAPH OF ANCHOVY FISHING

(From Blaxter and Hunter, 1982)

Optical detection would require a large signal-to-noise ratio to be effective. Contributing factors include the ambient light present in the atmosphere, the luminance of the water background, and the attenuation of light as it passes through the water. Light conditions vary from bright sunlight to a completely overcast night, causing a corresponding increase in the effectiveness of the LLLII. The amount of luminescence in the water background is important for a similar reason, since the contrast between the ambient light and the bioluminescence will determine what is detected by the LLLII. The attenuation of light as it moves through the water depends on the angle at which the light ray strikes the water surface, the transmissivity of the water, and the distance that the light must travel. The probability for detection also depends on the degree of disturbance generated by the moving vessel. This disturbance is a function of the shape and speed of the vessel, as well of the vessel's movement in relation to wind, waves, and swell (Donaldson, 1982).

Brown (1970) compares the contrast sensitivity of various sensors, including the naked eye, binoculars, and night vision devices. Figure 2 (Brown, 1970) provides an example of how this information may be applied to an actual submarine in order to determine detection depth. Assuming optimal stimulation and direct overhead viewing, it is possible to detect this submarine to a depth of 100 m on an overcast night using a starlight scope, a night vision device (Brown, 1970). This is



Depth of a submarine providing adequate contrast for detection assuming optimal stimulation of bioluminescence and direct overhead viewing

FIGURE 2: APPLICATION OF CONTRAST DATA TO SUBMARINE DETECTION

(from Brown, 1970)

a concrete demonstration of the potential of bioluminescence as a detection tool.

In addition to detection systems, bioluminescence has significant application to recent Navy interest in optical underwater communications systems. These systems involve the use of high pulse repetition rate, high energy lasers to communicate with submarines from aircraft or satellites. Two possible systems currently under evaluation are the Submarine/Air Optical Communication System (SAOCS) and the Strategic Blue-Green Laser Submarine Communication System (Donaldson, 1982). Water has the greatest transmittance for light of wavelengths from 430 to 530 nm, and both systems will use a wavelength of 480 $\pm$ 20 nm in order to take advantage of these transmission properties. This is precisely the wavelength of peak light emission by bioluminescent organisms, dinoflagellates in particular. The close spectral correspondence indicates that bioluminescent emissions may cause significant interference by raising the background noise to a level that is unacceptable for detection. A further complication is that bioluminescence does not make a steady, predictable contribution to the background noise, since it occurs sporadically. An understanding of the intensity, spectral properties, and control mechanisms of bioluminescent light is necessary in order to evaluate its full impact on laser communications systems.

II. BACKGROUND ON BIOLUMINESCENCE

Bioluminescence, the emission of light from living organisms, is a widespread phenomenon, both in taxonomic and spatial terms. At least forty-one taxonomic orders in the animal kingdom contain luminescent organisms (Harvey, 1952). The most well-known luminescent organism is the common firefly, Photinus pyralis. Many other luminous terrestrial organisms exist, including bacteria, fungi, worms and insects. However, these terrestrial species do not compare in either number or variety to the luminous marine species. The spread of bioluminescence throughout the ocean is very extensive and it has been estimated that 70% of the species and 90% of the individuals below the photic zone are bioluminescent (Lynch, 1978).

Bioluminescence has been observed in every ocean and at all depths extending to 10.9 km (Lynch, 1981b). Surface luminescence provides the most striking demonstration of bioluminescence and has been attributed to relatively dense populations of dinoflagellates because of their abundance and wide distribution. Recently, this assumption has been questioned and a study by Swift, et al. (1983) in the Sargasso Sea found that zooplankton, including copepods, larvaceans, and ostracods, produced more flashes and more bioluminescent light than dinoflagellates. This would be important because the type and intensity of emitted light varies between different species and knowledge of the relative light contributions made by each would be essential to predicting the bioluminescence expected

in a particular area. Comprehensive listings of luminous organisms were available in several sources including Harvey (1952) and, most recently, Herring (1978). In this paper only marine organisms are discussed, with concentration on the dinoflagellates, in particular, Pyrocystis lunula.

A. Distribution

Marine bioluminescence is spread throughout the oceans, both horizontally and vertically. The ubiquitous nature of this phenomena makes it very difficult to form an accurate quantitative assessment of its occurrence. This difficulty is largely a function of the immense area and volume of water that must be surveyed. Most of the information available on the global distribution of bioluminescence in the oceans is therefore qualitative. Several studies on the occurrence of marine bioluminescence exist, and are based on visual sightings as compiled from shipping reports. In one of the first global studies, Smith compiled data from the Meteorological Logs and Records of the Voluntary Observing Fleet from 1920- 1930. More detailed investigations of bioluminescence in regional areas are from the in the Okhotsk Sea, the Arabian Sea, the Atlantic Ocean, and the North Sea (Lynch, 1981a). Turner (1966), Staples (1966), and Lynch (1978) provide the most recent summaries of the worldwide distribution of bioluminescence. The data for these surveys came primarily from merchant shipping reports.

In order to accurately evaluate this data several factors must be considered. The first was the influence of shipping

density. The frequency at which bioluminescence is detected will be greater in areas of high shipping density simply because of the greater number of observers, and does not necessarily reflect a real increase in bioluminescence activity. Both Turner (1966) and Lynch (1978) have attempted to eliminate this bias towards well-traveled sea lanes by including shipping density in their analyses. In determining the shipping density it was important to include those ships that did not report any bioluminescence as well as those that did. Another problem encountered with merchant shipping reports of bioluminescence, was that they were irregular. A ship may have failed to report a sighting either through indifference, or uncertainty as to how or where it should be sent. Finally, visual sightings were limited by the sensitivity of the human eye. Research with low light level sensors, as discussed in Section 2, frequently has detected bioluminescence displays that are invisible to the human eye. Therefore, accurate surveying of the distribution of bioluminescence was and still is limited by the lack of adequate observational coverage and the sensitivity of the sensors. A potential solution to this problem would be the use of low light level sensors in aircraft or satellites to provide global coverage (Lynch, 1981b).

This method has disadvantages as well; since there is no uniform disturbance of the observed area, a negative report may indicate either the absence of luminous organisms or the lack of sufficient stimulation. Current studies are underway to

determine the feasibility of using a laser on the airborne platform as a means of stimulating bioluminescent activity (Lynch, 1982). Determination of the vertical distribution of marine bioluminescence is uncertain and corresponds to the relatively few underwater observations available. Bathyphotometers are used to measure light under the water surface and may be towed or stationary. The development of automatic bathyphotometers that are similar to current meters is underway and could increase greatly the amount of data available. At the present time, the only feasible approach to this problem is through detailed analysis of small areas and application of the generated data to larger regions.

Despite these limitations on quantitative measurements, it is valuable to use the available data to describe the worldwide distribution of bioluminescence. Bioluminescence exists in every ocean from the tropics to the poles. Surface luminescence is mainly from dinoflagellates and other plankton and its occurrence depends on their distribution. The highest concentrations of these organisms are in shallow coastal waters and in the nutrient enriched areas associated with upwelling. The Indian Ocean contains the greatest amount of bioluminescence, particularly in the Arabian Sea and the Persian Gulf. The Strait of Gibraltar, at the entrance to the Mediterranean, is another highly luminous area. In the Pacific and Atlantic Oceans, maximum brightness usually occurs along the coast when increased nutrient concentrations cause dense population growth or "blooms" (Lynch, 1981a). The Caribbean

Sea has a relatively high and generally constant level of luminescent activity, with particularly high levels found in the shallow bays of Puerto Rico and Jamaica (Lynch, 1981a).

While the most striking displays of bioluminescence are found on the surface, the phenomenon has been observed at nearly every depth in the ocean. Bioluminescence has been observed in the Japanese Trench at a depth of 7,200 m and near the ocean floor at a depth of 10.9 km in the Challenger Deep by the bathyscaph TRIESTE (Lynch, 1981b). The first recording of luminescence at various depths was made in 1934 from a bathysphere (Lynch, 1981b). Quantitative measurements were not made until the development of bathyphotometers, from which most information on vertical distribution is currently generated. Most luminescence is found in the euphotic zone, where the penetrating sunlight supports a relatively dense population Much of the early work with vertical distribution was concerned with establishing a connection between bioluminescence and the deep scattering layer. No definite correlation with the deep scattering layer was found, but a lumnescence maximum has been associated with the thermocline (Tett and Kelly, 1973) and abrupt changes in luminescence have been connected with sharp temperature changes (Lynch, 1981b).

The vertical distribution of bioluminescence varies greatly with time, location, and season. Generally, luminescence is greatest in the euphotic zone, after which it decreases abruptly and then increases to a secondary maximum, usually between 200-1000 m (Tett and Kelly, 1973). A

relationship exists between the distribution of plankton and luminescence; however, this relationship is very complex and has not been explained completely. Three commonly observed features of the distribution of luminous organisms are that they form a maximum in the euphotic zone, they often form layers with depth, and they exist in decreasing densities below 1000 to 2000 m (Tett and Kelly, 1973).

Surface luminescence occasionally occurs in spectacular displays of uncertain origin. These displays are mostly in the Indian Ocean and, though they have not been closely investigated by scientists, the large number and similarity of the reports confirms their existence. One phenomenon, called "milky seas", occurs as a steady, diffuse glow over a relatively large area. The most frequent observations of this are during times of high productivity and it may be the result of continuously glowing bacteria growing on slicks of decaying algae (Tett and Kelly, 1973). "Phosphorescent wheels" are another unusual display that appear as waves or beams moving across the water at high speeds. While the "wheels" are generally a shallow water phenomenon, other displays known as "erupting balls" are usually found in deep water. These occur as patches of light appear to shoot to the surface, spread out into circular patches, and then gradually fade away (Tett and Kelly, 1973). Possible explanations for these displays include seismic events, distortion of the earth's electromagnetic field, and optical illusions (Lynch, 1981b). Other unusual displays are streaks of luminescence in association with moving

searchlight or radar beams (Lynch, 1981b). There is no definite correlation of cause and effect for any of these phenomena.

B. Kinetics of light emission

A great diversity of luminous marine organisms exists, from the small bacteria and dinoflagellates to large species of fish and squid. These organisms may be divided into categories based on the control they exert over their light emission. In the smaller marine organisms, such as the dinoflagellates, the entire organism may flash or glow in response to a stimulus. Other organisms, including the ostracod, a planktonic crustacean, secrete chemicals that react to form an extracellular glow. The ostracods secrete two granules with a mucus emission that react to form a luminous cloud around the organism (Boden and Kampa, 1974). In more complex organisms, the emission of light is controlled by light organs, known as photophores. These photophores contain either symbiotic luminous bacteria or specialized cells, known as photocytes, that contain the chemicals needed for luminescence. Photophores control the emission of light through physical means that range from simple membranes to complex arrangements of lenses and reflecting surfaces. The location of the photophores varies from one organism to another depending on their intended use.

C. Functions of Bioluminescence

The emission of light would have important functions and is of favorable adaptive significance to bioluminescent marine organisms. This is shown by the widespread taxonomic distribution of the phenomenon as well as by the many physiological adaptations found in luminescent organisms. Some early researchers, including Harvey (1952), suggested that the biochemical effects of bioluminescence were more important than the emission of light. This argument was refuted by Tett and Kelly (1973). The energy loss associated with luminescence would not allow the organism to survive selection if it did not provide some selective advantage. Many different functions of bioluminescence have been proposed, and they may be classified by their application to offense, defense, or communication (Donaldson, 1982).

The offensive use of dim luminescent photophores to attract prey would allow the predator to conserve energy. This would be of particular benefit at greater depths, where fewer prey are found and greater distances must be traveled to reach them. This function is found in several species of fish, with the angler fish as one example. The angler fish uses a photophore on its skull to attract prey to its mouth. Also, some predators may use flashes of light to confuse prey just before capture, but this behavior has not been well documented (Tett and Kelly, 1973).

Bioluminescence is more common as a defense mechanism and specific functions include distraction, masking, and

concealment. Sharp, bright flashes startle or temporarily blind the predator, allowing the prey to escape during the distraction. Masking, or camouflage, is accomplished often by counter-illumination, in which the organism becomes luminous to match the downwelling light and prevent silhouetting from above. Counter- illumination has been studied in the shrimp Sergestes similis (Warner, et al., 1979) and in two species of squid (Young and Mencher, 1980). In both cases the organism matched both the intensity and spectral characteristics of the downwelling light and was capable of responding to relatively short-term changes. Also, as another form of defense, the organism may secrete a luminous cloud to conceal it from the predator while it escapes.

Communication is another important function of marine bioluminescence, especially among members of the same species. No marine organism exhibits the well-defined relationship of flashing signals and mating found in the firefly. However, there is a suggestion that luminescence may be involved in intraspecific recognition among certain fish and euphausiids and help the organisms to swarm during the breeding period (Tett and Kelly, 1973). Luminescence may also serve to attract potential mates among some species of fish (Harvey, 1952). Although some use of bioluminescence as a means of communication is likely, none has yet been conclusively established in marine organisms.

D. Dinoflagellates

Dinoflagellates have long been regarded as the principal cause of bioluminescence in the euphotic zone. Recently that assumption has been questioned (Swift, et al., 1983), but, even if not the largest, they are still a major contributor to the luminescence in the oceans. The dinoflagellates are found in the phylum Protozoa and include a large variety of planktonic organisms, some exhibiting plant-like characteristics, while others behave like animals. Many of them are known to be photosynthetic to some degree and they are among the most abundant species in the euphotic zone. There are two suborders, Adinida and Dinifera. Pyrocystis lunula, along with most dinoflagellates, belongs in the Dinifera.

There are both luminescent and non-luminescent species of dinoflagellates and within some species, both luminescent and non-luminescent varieties have been observed (Tett and Kelly, 1973). While considerable variations in morphological characteristics exist among different species, all dinoflagellates share certain structural features. The dinoflagellates have two flagella, one lying in a groove along the main axis of the cell, and the other along a transverse groove. They are motile for part or all of their life-cycle, depending on the species. The cell nucleus is large and the cell is often surrounded by a plated wall composed of cellulose (Donaldson, 1982). Those species having the wall are referred to as "armored", while those without are either "unarmored" or "naked". Reproduction is generally asexual, involving binary

fission where the parent divides to form two identical daughter cells.

The Pyrocystis species were first reported by the Challenger expedition of 1873-1876 as the chief source of phosphorescence in equatorial waters. This observation has been supported by subsequent data indicating that Pyrocystis species are the most abundant large dinoflagellates in tropical and subtropical waters (Swift, et al., 1973). Pyrocystis species are relatively large, unicellular dinoflagellates and exhibit plant-like characteristics. Cells of Pyrocystis species have a thin cell wall of cellulose fibers and are "unarmored" or "naked" (Swift and Durbin, 1971). The large size and large population densities of this species make it a significant source of marine bioluminescence and facilitate laboratory observations. Another indication of the importance of Pyrocystis is that it has been found that some of these species produce from 100 to 10,000 times as much light as other photosynthetic dinoflagellates (Swift, et al., 1973). For these reasons, as well as its availability in culture, Pyrocystis lunula is the organism for this experiment.

Pyrocystis lunula cultures are photosynthetic and require a minimum of six hours of sunlight daily to live (Hickman and Lynch, 1981). In the ocean, they tend to concentrate near the bottom of the photic zone (Swift and Meunier, 1976). Peak light emission occurs at 477.5 $\pm$ 1 nm (Swift, 1967), with a stimulable half-life of 30 minutes (Biggley, et al., 1969).

There are three stages in the life cycle of Pyrocystis lunula. The adult stage has a crescent-shaped cell, approximately 100 μm in length, that lacks flagella and is non-motile (Figure 3). This is the lunate cyst and it is brilliantly luminescent with the light originating from the nuclear area in the center of the cyst (Swift and Taylor, 1967). These crescent-shaped cells reproduce asexually and form one, two, or occasionally four new cells that form the second stage in the life cycle. These smaller cells, called "swarmers", contain a poorly developed transverse groove, lacking a flagellum. They may, however, have a single flagellum trailing the cell (Swift and Durbin, 1971). These reproductive cells swim sluggishly for 5 to 10 seconds before losing their flagella and swelling to form a new lunate cyst. The swarmers are not bioluminescent and their function is not certain (Swift and Taylor, 1967). The third stage of the life cycle is a large spherical cyst of up to 200 μm in diameter that is rarely observed. These large cells subsequently divide to form the crescent-shaped cells (Swift and Durbin, 1971). Swift and Durbin (1971) describe the life cycle in greater detail.

E. Light Generation

As a physical phenomenon, luminescence is the generation of light without heat. When analyzed from a chemical standpoint, it is the result of a reaction in which the liberated energy excites a product molecule rather than being released as heat. The excited molecule then releases this energy as photons as it returns to normal energy state (Boden

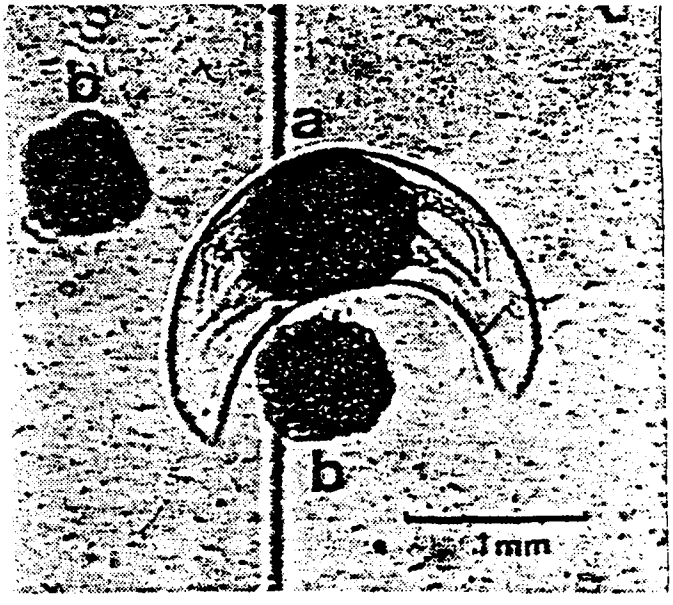


FIGURE 3: 425X MAGNIFICATION OF (a) PYROCYSTIS LUNULA

(from Donaldson, 1982)

and Kampa, 1964). Bioluminescence takes place when the chemicals used for this reaction develop in living cells and the reactions are monitored by a biological catalyst. The specific molecular reactions leading to bioluminescence are of five different biochemical types (Boden and Kampa, 1974). These are listed in Table 1 along with representative marine organisms and peak light emissions for each.

The five reactions may be classified into two distinct bioluminescent systems: soluble and particulate. The first four types in Table 1 are soluble reactions, referred to as enzyme-substrate oxidations. The substrate, known as luciferin, is oxidized in the presence of an enzyme catalyst, identified as luciferase. The terms luciferin and luciferase come from Dubois (1887) and have since become synonymous with "enzyme-substrate" terminology.

Luciferin is a molecule of relatively low molecular weight and is the active molecule in the luminescent reaction. The chemical structure of luciferin varies in different species. The luciferin molecule found in P. lunula is common to all dinoflagellates. It has a molecular weight between 550 and 600 and is very susceptible to air oxidation (Dunlap, et al., 1981). As with luciferin, there are several types of luciferase that are available, depending on the organism involved. Dinoflagellate luciferases are large proteins with molecular weights of approximately 130,000. Active fragments of the luciferase protein, with molecular weights of approxi-

TABLE 1. TYPES OF BIOCHEMICAL REACTIONS LEADING TO LUMINESCENCE

		Representatives	Peak emission nm
Type I	Direct oxidation: simple enzyme-substrate systems $LH_2 + O_2 \rightarrow$ light	Pholas	485 nm
		Cypridina	460 nm
		Apogon	460 nm
		Gonyaulax	470 nm
Type II	Substrate activation followed by oxidation: adenine nucleotide linked $\text{pre-}LH_2 \xrightarrow{\text{activation}} LH_2 \xrightarrow{O_2} \text{light}$	Renilla	460 nm
Type III	Substrate reduction followed by oxidation; pyridine nucleotide linked $L \xrightarrow{DPNH} LH_2 \longrightarrow \text{light}$	Bacteria	490 nm
Type IV	Peroxidation reactions $LH_2 + H_2O_2 \longrightarrow \text{light}$	Balanoglossus	480 nm (?)
		Chaetopterus	460 nm
Type V	Ion-activated: "pre-charged" systems $P \xrightarrow[\text{or } H^+]{Ca^{++}} \text{light}$	Gonyaulax	470 nm
		Aequorea	460 nm

(From Boden and Kampa, 1972)

mately 35,000, will catalyze the reaction as well (Donaldson, 1982; Dunlap, et al., 1981).

The general formula for the reaction is shown in Figure 4. For each particular luciferin, a specific luciferase is required to catalyze its oxidation. While molecular oxygen is usually the oxidant, H_2O_2 is used in some reactions (Nealson, 1981). High salt concentrations are required for the reaction to proceed (Fuller, et al., 1972). The concentration of hydrogen ions is involved also in the activation of luminescence. A more complete description of the luciferin-luciferase reaction is found in Hastings (1978).

The luciferin-luciferase reaction produces light in an extremely efficient manner. As shown in Figure 4, one quantum of light is emitted per molecule of substrate oxidized. Many chemiluminescent systems exist, but their reactions have very low efficiences in terms of the amount of light produced. The reason for this difference in light production efficiency is traceable to the role of the enzyme catalyst (luciferase) in bioluminescent reactions (Cormier and Totter, 1968).

The second type of luminescence system, the particulate system, is represented by the fifth type of biochemical reaction in Table 1. It involves a non-soluble, crystalline-like particle, first identified as a "scintillon" by Desa, et al. (1963). This particle emits light when the hydrogen ion concentration is increased (optimum pH of 5.7) in the presence of oxygen (Fuller, et al., 1972). After it is activated the scintillon may be recharged by increasing the pH.

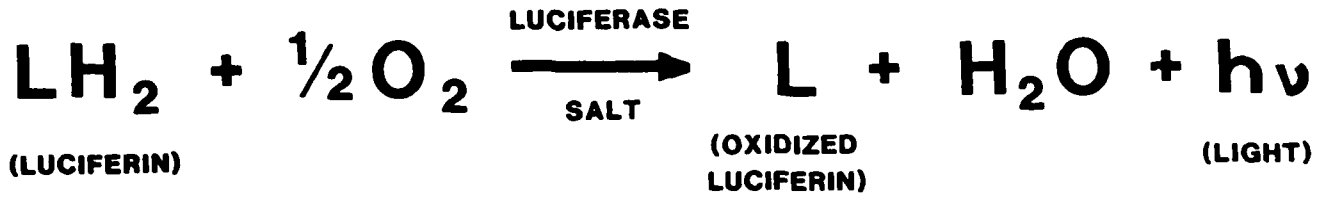


FIGURE 4: GENERAL LUMINESCENCE REACTION

The detailed mechanism that produces light in scintillons is not known presently.

While the soluble system generates a long-lasting glow, the particulate system generates a single flash of light in response to activation of the scintillon. This flash approaches the fast emission characteristics observed during mechanical stimulation (Fuller, et al., 1972). The glow caused by the soluble system is diffuse and spread over a large part of the organism. The scintillons, on the other hand, are much more localized and exist as discrete microsources. The light generated by dinoflagellates exhibits the characteristics of either one of these systems or a combination of both. In Pyrocystis lunula the soluble system is predominant.

F. Stimulus-Receptor Mechanism

The control of bioluminescence on a molecular level is accomplished by the chemical reactions discussed in the previous section. This subcellular system must be activated by outside forces. The organism uses physiological control to transmit the impact of the environment to elements within the cell. The response to stimulus in dinoflagellates is an all-or-none reaction that occurs when an action potential is reached. Eckert (1966) observed that "The luminescent flash is all-or-none. It potentiates, summates, and fatigues independently of changes in the amplitude of the action potential ..." Extensive studies of Noctiluca have shown that mechanical stimulation will induce gradually increasing potentials that depend on the strength of the stimulus (Eckert,

1966). One interpretation of the physical manifestation of this stimulus is a shear produced by differential water movement at the surface of the cell (Dunlap, et al., 1981). The receptor mechanism may also be sensitive to other physical forces, such as a change in pressure. If the stimulus is strong enough, the receptor mechanism on the cell surface will trigger an action potential across the cell membrane. This action potential will then change the electrical potential of the cell membrane, allowing protons into the cell. The increased hydrogen ion concentration will trigger luciferin oxidation and light production. Once started, the action potential propagates along the membrane, with a speed measured in Noctiluca of 60 μm/ms, stimulating luminescence in its path (Dunlap, et al., 1981).

The receptor mechanism, that senses the physical stimulus and activates the membrane, is not yet understood. One proposed mechanism depends on the ionic environment, specifically the presence of Ca++ ions. It suggests that a physical force causes a temporary local distortion of the cell membrane and increases the permeability to Ca++ ions. The Ca++ ions then trigger an increase in H+ ions and subsequent light production (Hamman and Seliger, 1982). It has also been suggested that the receptor mechanism may involve an organelle containing trichocysts suspended by delicate fibers that would be sensitive to mechanical stimuli (Tett and Kelly, 1973). This area requires much further study in order to determine the actual mechanism.

G. Control Factors

The relationship between the molecular and physiological elements controlling bioluminescence is represented by the model in Figure 5 (Dunlap, et al., 1981). Physiological control involves the organism's interface with the environment and includes the receptor mechanism, the effects of light, and the circadian rhythm. Molecular control involves the actual chemical reactions that lead to light production. In general, the molecular system supplies the potential for bioluminescence, while the physiological control system determines when and how that potential is activated. Other factors, such as nutrients and temperature, influence the expression of bioluminescence as well, primarily through their effect on the growth of the luminous organisms. The molecular control and receptor mechanism have been discussed at length in sections E and F. The remaining control factors are discussed below.

1. Light

Light has a considerable effect on the bioluminescent capability of photosynthetic dinoflagellates in two, apparently contradictory, ways. Photosynthesis is required for the organism to survive. Pyrocystis lunula, for example, requires a minimum of six hours of light daily. Laboratory cultures are normally placed on a 12-hour light and 12-hour dark cycle to reproduce the diurnal light alternation of the environment. The light phase is referred to as the photophase, while the

Elements controlling the bioluminescent output of luminescent photosynthetic dinoflagellates. Arrows point from the controlling element to the structure or process being controlled. The probable involvement of ions is noted in several places.

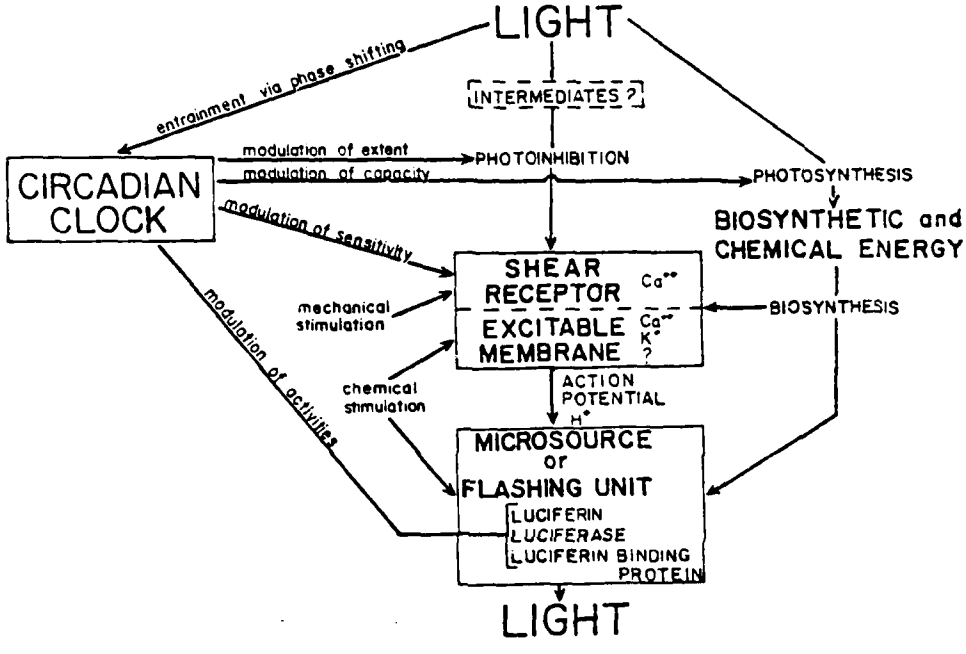
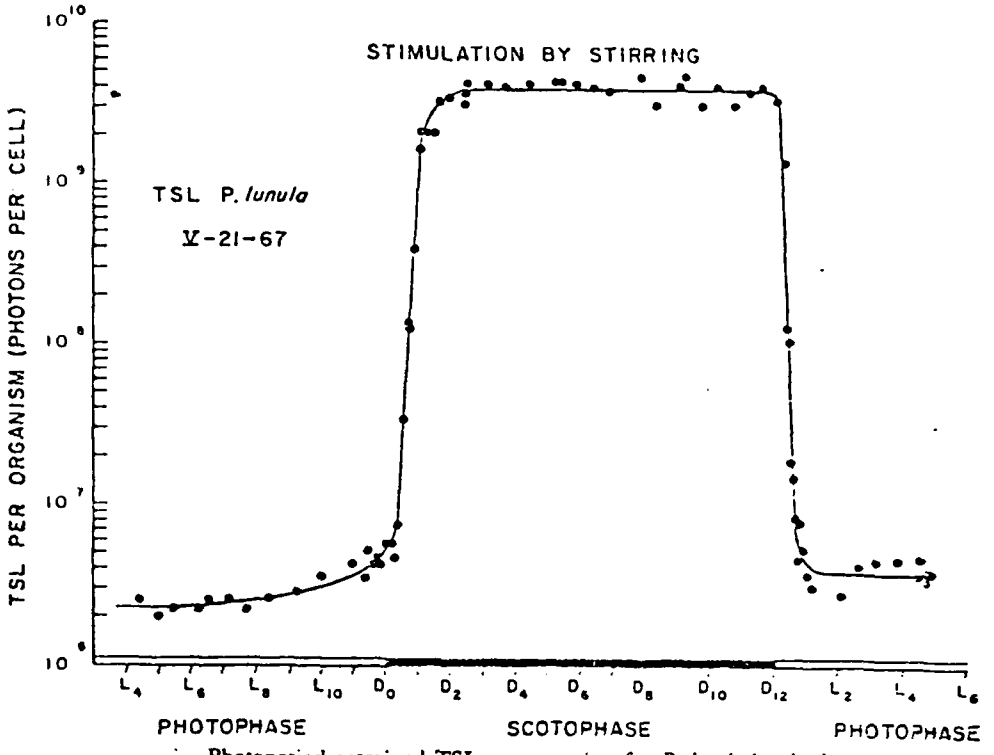


FIGURE 5: MODEL OF LUMINESCENT CONTROL ELEMENTS

(from Swift, 1967)

dark phase is called the scotophase. The degree of luminescence at night is proportional to the amount of light the organism receives during daytime illumination (Sweeney, 1981). Cultures of Pyrocystis lunula left in continuous darkness were found to lose 60% of their total stimulable light (Swift and Meunier, 1976). It has been suggested that the relationship of luminescence and photosynthesis depends on the structure of the luciferin molecule. Luciferin has been identified as a polypyrrole derivative and it may be a derivative of the most abundant marine polypyrrole, chlorophyll, which has an important role in photosynthesis (Dunlap, et al., 1981).

Exposure to light also acts to suppress light output through an effect known as photoinhibition. This effect causes a marked decrease in stimulable bioluminescence during the organism's photophase (light phase). Ratios of 950:1 for laboratory samples and 4000:1 for natural populations have been found between scotophase and photophase bioluminescent capacity (Biggley, et al., 1969) and Figure 6 illustrates this sharp difference (Swift, 1967). During the first 10 to 30 minutes of the scotophase, there is little change in stimulable bioluminescence, but then there is a sharp exponential increase that reaches a maximum three hours into the scotophase. Throughout the remainder of the scotophase the level remains close to this maximum point. After 5 to 10 minutes in the photophase, there is an exponential decrease to a minimum level that is maintained until the next scotophase (Swift, 1967).



Photoperiod-entrained TSL per organism for *P. lunula* in absolute units of photons over a complete photoperiod.

FIGURE 6: DIFFERENCE IN BIOLUMINESCENT CAPACITY BETWEEN SCOTOPHASE AND PHOTOPHASE

(from Biggley, et al., 1969)

Photoinhibition results in a decreased response to mechanical stimulus, as well as a decrease in total bioluminescent capacity (Hamman and Seliger, 1972). It affects the mechanical stimulus response system by inducing a hyperpolarization of the cell membrane (Hamman and Seliger, 1982). This increases the threshold of sensitivity, just as membrane depolarization and admission of hydrogen ions reduced the threshold. The spectrum of incident light causing photoinhibition peaks at 562 nm. This is a significant displacement from the peak of the emitted light and prevents the organism from inhibiting itself or other organisms (Esaias, et al., 1973). Photoinhibition may be mimicked or reversed chemically by the removal or addition of Ca++ ions (Hamman and Seliger, 1972). The relation of photoinhibition and photosynthesis is evident because all the photosynthetic dinoflagellates photoinhibit, while none of the non-photosynthetic species do. A possible reason for photoinhibition is that it could conserve energy when the ambient light is strong enough to make bioluminescence an ineffective defense (Esaias, et al., 1973). It has not been determined why this would not occur in the non-photosynthetic species as well.

2. Circadian Rhythm

In addition to the variation of light emission with the day-night environment light cycle, luminescent organisms also demonstrate a circadian rhythm in many of the processes associated with luminescence. The circadian rhythm refers to a

biological clock that paces all life processes. The effect of this rhythm on light emission was first shown by Sweeney and Hastings (1957) in Gonyaulax polyedra. Circadian rhythms have also been found in the activity of luciferin, luciferase, scintillons, and in the sensitiviy to mechanical stimulation (Donaldson, 1982). The amplitude of the stimulable bioluminescence decreases with each cycle as an organism is held in continuous darkness. However, the cyclic nature is still evident as shown in Figure 7 (Swift, 1967). Figure 5 shows how the circadian clock modulates the various aspects of luminescence. The circadian rhythm should not be confused with the diurnal response to day-night variations in light intensity.

As shown in Figure 6 (Swift, 1967) the diurnal alternation in light intensity induces a change in the bioluminescent capacity that most closely resembles a square wave form, with sharp changes in amplitude, and maximum and minimum values that are relatively constant over the entire phase. The change in luminescence caused by the circadian rhythm follows a sinusoidal, rather than square, wave pattern, as shown in Figure 7. Maximum sensitivity occurs in the middle of the scotophase. Light does influence the circadian rhythm, as it shifts the phase of the pattern (Donaldson, 1982).

The circadian rhythm has a very pronounced effect on light emission that must be taken into account when laboratory measurements are made. In order to make valid comparisons between two cultures, the measurements should be made at the same point in the circadian rhythm. Since cultures are often

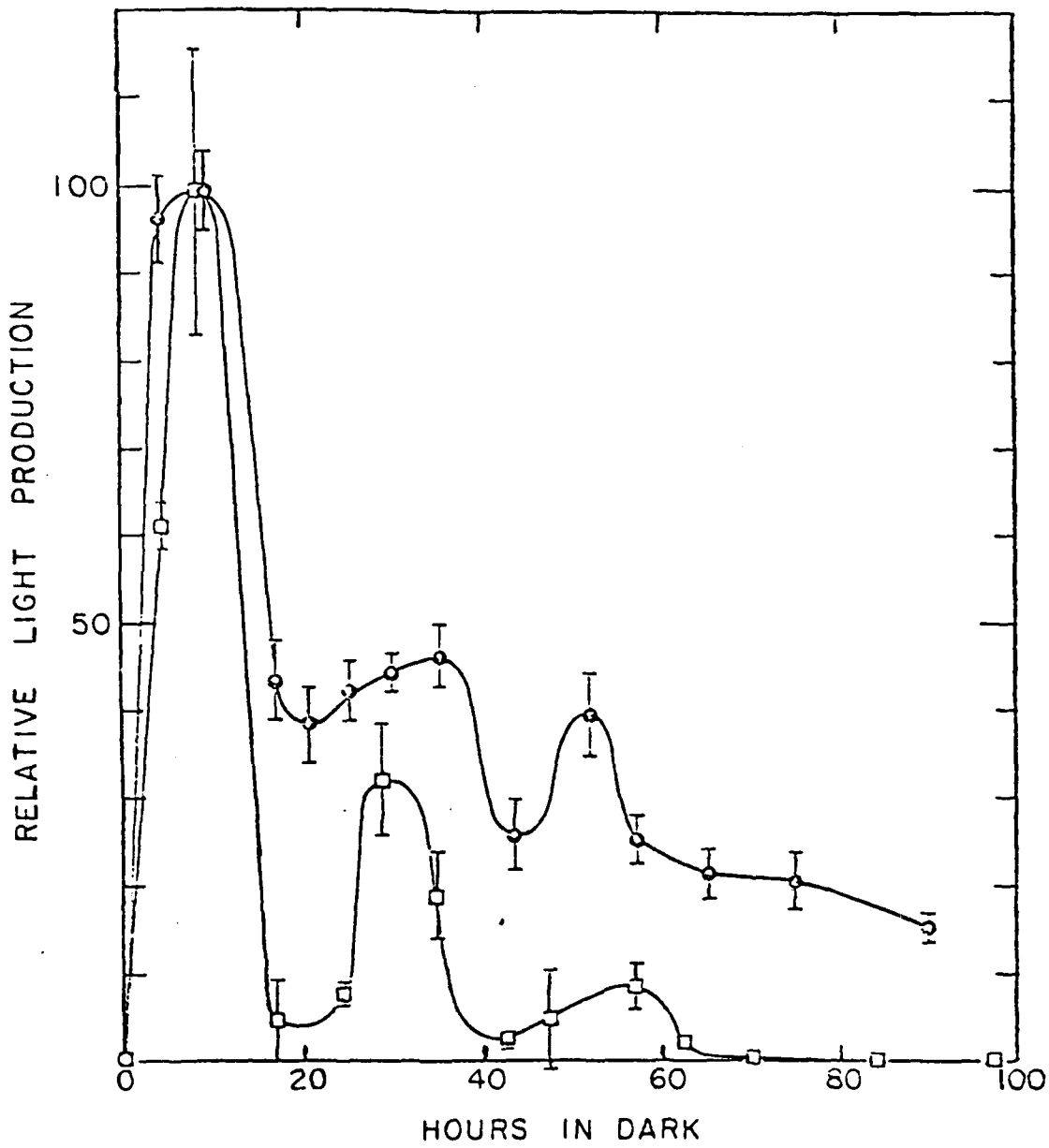


FIGURE 7: DEMONSTRATION OF CIRCADIAN RHYTHM IN CONTINUED DARKNESS

(from Swift, 1967)

grown on light cycles at different local times, another reference point should be designated. The time is referenced to the diurnal cycle of the organism and customarily starts with the beginning of the light cycle. This point is designated as CT 0000, where CT stands for circadian time. Since maximum luminescence occurs in the center of the scotophase (CT 1800), that is the optimal time to perform experiments (Sweeney, 1981).

3. Temperature

Dinoflagellates are found in every ocean and exist over a broad range of water temperature. The greatest amount of bioluminescence, in terms of both intensity and frequency, is found in tropical and sub-tropical waters. Warming of the water is associated usually with an increase in luminescent organisms. The increase in luminescence in both of these cases results from the increasing dinoflagellate population, rather than a temperature induced change in the light production of an individual organism. Low temperatures have been shown to decrease the rates of cell division without decreasing the bioluminescent capacity (Swift, et al., 1981). Surprisingly, an inverse relationship between temperature and luminescence was discovered by Sweeney (1981). As the temperature in Gonyaulax cells, cultured at 20°C, was decreased from 20° C to 13°C, the stimulable bioluminescence increased linearly. Below 13°C, bioluminescence occurred spontaneously until the cell reached exhaustion. For cultures grown at 10°C, this increase

in luminescence was not observed, indicating that the temperature history of the organism influenced its luminous characteristics (Sweeney, 1981).

4. Nutrients

Many factors moderate the occurrence of bioluminescence in an organism, either directly or indirectly. Nutrients exert an indirect influence on bioluminescence that stems from their impact on dinoflagellates growth. In nutrient-enriched waters, such as those found along the coast and in areas of upwelling, large concentrations of dinoflagellates develop. These large concentrations often cause intense bioluminescent activity. Experiments relating the luminescence per cell to nutrient level have found an unexpected lack of correlation (Sweeney, 1981). Nitrate and phosphate, two of the most needed nutrients, were used and, while their absence limited growth, it did not reduce the bioluminescent yield per cell. Experiments with iron sequestrine demonstrated that it also had very little effect on bioluminescence (Sweeney, 1981). The obvious independence of bioluminescence from external nutrient concentrations indicated that this process was maintained at the expense of others in limited nutrient conditions. This provided further evidence that bioluminescence fulfills an important function.

5. Additional Control Factors

The control of bioluminescence is dependent on many factors affecting different aspects of the luminescent process or the organism itself. The major variables have been identified and discussed, but a myriad of less significant factors also exert direct or indirect control.

The growth stage of the culture has been shown to affect light emission from individual organisms. A slight reduction, less than a factor of ten, in the luminescence per organism was observed for old, dense cultures, as compared to those in a logarithmic growth phase (Sweeney, 1981). However, another study found that the total stimulable light per cell was nearly constant from the exponential through stationary phases of growth (Biggley, et al., 1969). To eliminate this as an experimental variable, cultures used for testing are generally maintained in the logarithmic phase of growth.

The concentration of organisms is an obviously significant factor in determining the total amount of light production. Any factors that increase or decrease dinoflagellate populations may be treated as bioluminescent control factors (Section G.4.). The sporadic occurrence of dense blooms of dinoflagellates, known as "red tides," in warm coastal waters, are accompanied frequently by intense bioluminescent displays. The cause of these blooms has not been fully explained. In tropical waters, a combination of topographical and meteorological effects may concentrate dinoflagellates in "bioluminescent bays". Unlike the "red tide" phenomenon, these persist over an extended period of time and result from the

concentration of warm saline water to which the dinoflagellates migrate and where they reproduce rapidly (Seliger, et al., 1970). Seasonal variation of dinoflagellate populations occur as the result of changing sunlight and nutrient availability. The lack of sunlight limits winter growth, but in the spring a bloom occurs in response to increased sunlight and accumulated nutrients. The population falls once the excess nutrients are used and remains limited until fall storms churn nutrients to the surface, causing a secondary, less intense bloom (Brown, 1970). The dinoflagellates also respond to diurnal variations in sunlight, moving towards the surface at night and deeper during the day. This is an attempt to maintain a constant light environment (Boden and Kampa, 1974).

A correlation between salinity and bioluminescent activity has been demonstrated. Salinity affects dinoflagellate blooms, and waters of high salinity, such as the Red Sea, are often highly bioluminescent as well (Donaldson, 1982). Variations in salt concentrations of the growth medium have shown that a balanced salt content corresponding to seawater is necessary for optimal luminescence (Harvey, 1952).

The degree and duration of previous stimulation experienced by the organism also affects its capacity for light emission. Fatigue and the cessation of light emission occur after repeated stimulation, depending on the magnitude and duration of the stimulus. Higher rates of stimulation will induce fatigue sooner, but since the light output in each flash

is greater, the total light emitted remains constant (Widder and Case, 1981).

6. Controlled Stimulation

Laboratory study of bioluminescence requires a means of stimulating light emission. In order to obtain meaningful data that may be compared to previous or subsequent experimental results, the researcher should ensure that the stimulus is repeatable and that the experiments actually measure what they intend to. Several means of stimulation are discussed in Harvey (1952), including electric currents, heat, salt concentrations, narcotics, and light. Research has concentrated on mechanical and chemical stimuli, and recently on laser stimulation.

Mechanical stimuli have been used most extensively and include bubbling air, stirring, pumping samples through an impeller tube, and pressure pulses. The first three are disadvantageous in that it is difficult to precisely control the amount of stimulation. Another problem existing with an impeller tube is that the equipment may stimulate the organisms before they are observed. This could happen as the sample passes through piping prior to entering the observation chamber. These tubes are most effective when used to stimulate cells to exhaustion. Pressure stimulation, on the other hand, allows a more exact measurement of luminescence, as intermediate stages as well as at the start and endpoints may be

measured. The effects of pressure on bioluminescence have not been closely investigated. Previous experiments were generally conducted at high pressure and had mixed results. However, Donaldson (1982) demonstrated that slight pressure changes may be used to stimulate bioluminescence.

Studies by Hickman, et al. (1981) and Hickman and Lynch (1981) have shown lasers to be an effective bioluminescent stimulus. The light output increased with the intensity of the laser energy. Optimal response occurred at a laser wavelength of 585 nm. Stimulation by an ultrasound frequency of 880 kHz has been used successfully as well (Filimonov and Sadovskaya, 1982). Both laser and ultrasound stimulation are very useful, since, like pressure pulses, they may be precisely controlled and easily repeated.

The close involvement of ions in the mechanical stimulation of bioluminescence indicates that chemical stimulation is an effective means of inducing bioluminescence. Chemical stimulation bypasses the mechanical system by delivering protons directly to the membrane. Studies with Pyrocystis fusiformis have found that the most effective ions are Ca++, K+, NH4+, and H+. Chemicals involved in Ca++ transport or binding were also found effective (Hamman and Seliger, 1972). Acid stimulation has a similar effect since it involves changing the hydrogen ion concentration. In Figure 8 (Widder and Case, 1981) the effect on luminescence of both mechanical and acidic stimulation is portrayed. Also shown is the fact that further luminescence can be mechanically stimulated even

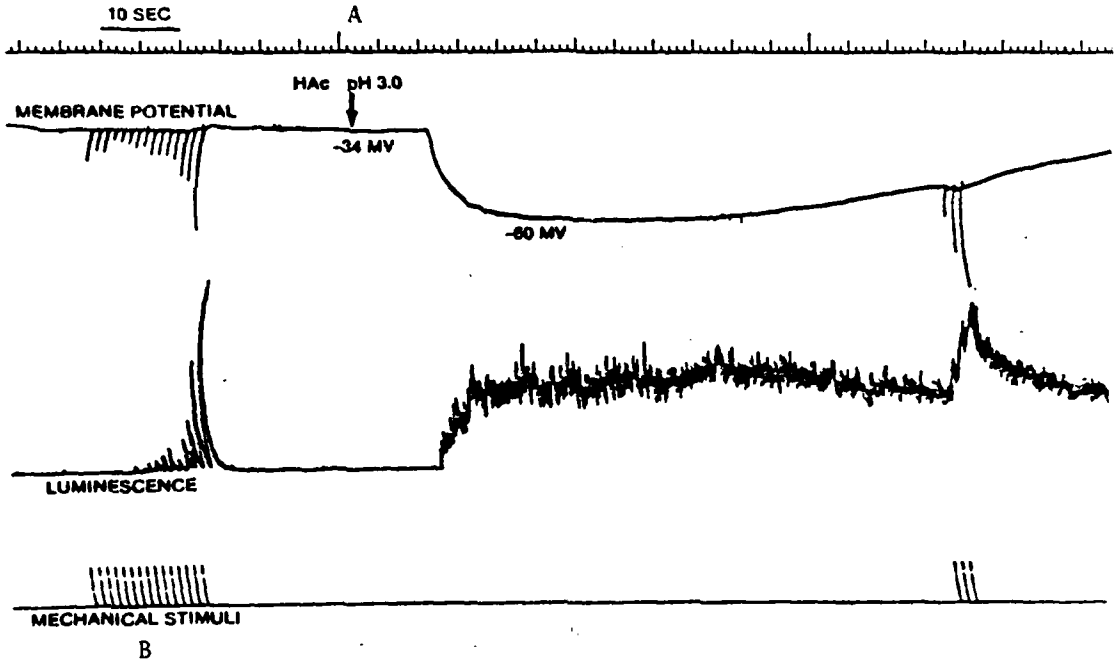


FIGURE 8: MEMBRANE POTENTIAL AND LUMINESCENCE DURING ACID (A) AND MECHANICAL (B) STIMULATION

(from Widder and Case, 1981)

during acidic stimulation. This supports the contention that chemical stimuli bypass the mechanical system and supply protons directly to the subcellular particles.

H. Emitted Light

The light emitted from dinoflagellates falls predominantly in the blue-green spectrum and may be divided into four general types. These are a nearly constant dim glow, a spontaneous flash, a flash in response to a stimulus, and a steady "death glow". The dim glow is not normally visible to the naked eye, but may be observed through amplification with a photomultiplier tube. Spontaneous flashes are relatively rare in Pyrocystis lunula. The "death glow" is a relatively constant output of light resulting from cell damage as the organism dies (Biggley, et al., 1969). Dinoflagellate response to stimulation occurs after a slight delay, usually 15 to 20 ms. The flash, shown in Figure 9 (Donaldson, 1982), has a rapid rise-time of between 30 and 40 ms, followed by a slower, exponential decay lasting approximately 500 ms (Hickman and Lynch, 1981). The amount of light available from a Pyrocystis lunula flash has been measured as 4x10^9 photons/cell and 2x10^12 photons/cm^2 at the edge of the cell (Hamman, et al., 1981).

The spectral characteristics of all dinoflagellate flashes are nearly identical, indicating that the excited product molecule is the same in each case (Seliger, et al., 1969). The spectrum ranges from 430 to 580 nm with an average peak emission at a wavelength of 480 nm (Tett and Kelly, 1973). The

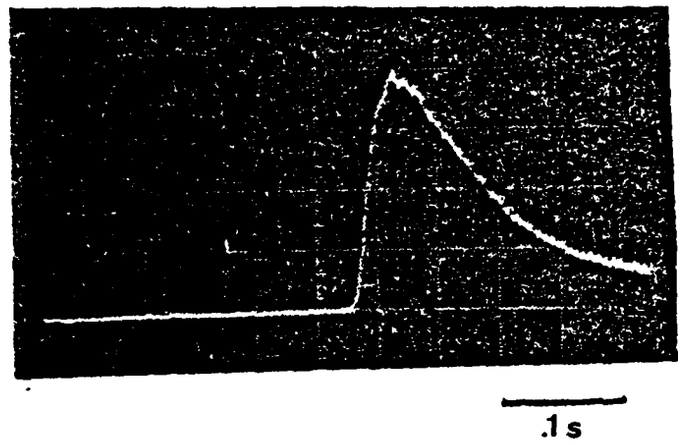


FIGURE 9: PYROCYSTIS LUNULA FLASH FOLLOWING MECHANICAL STIMULATION

(from Donaldson, 1982)

peak emission wavelength for Pyrocystis lunula occurs at 477.5 ± 1 nm (Swift, 1967). As shown in Figure 10 (Swift, 1967), the emission spectrum is skewed toward longer wavelengths. The wavelength of peak light emission corresponds to the wavelength of maximum sea water transparency. It is also near the maximum sensitivity of the dark-adapted human eye (Donaldson, 1982). In addition, the eyes of marine organisms are generally sensitive to this same spectrum. These closely corresponding spectral characteristics demonstrate the importance and possible use of bioluminescence in detection and communication systems.

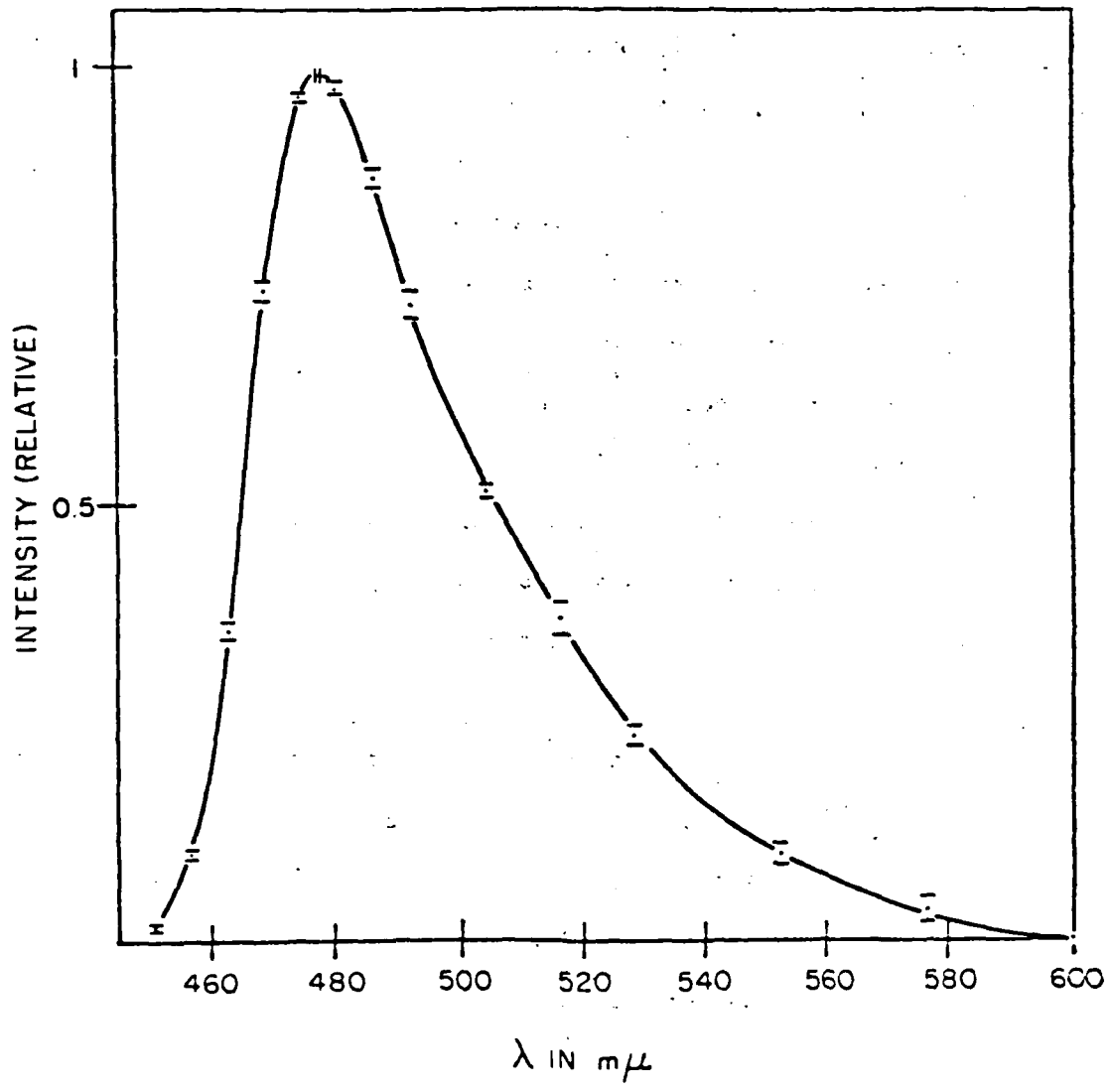


FIGURE 10: PEAK EMISSION CHARACTERISTICS OF PYROCYSTIS LUNULA

(from Swift, 1967)

III. MATERIALS AND METHODS

A. Culture

A culture of Pyrocystis lunula was obtained from A. Stiffey at the Naval Research Laboratory, Washington, D.C. It was originally cultured by E. Swift in 1964 from a sample, identified as T-37, taken off the coast of Brazil (Lat 30°S, Long 33°W). The culture was grown in the "F/2" enriched seawater formula of Guillard and Ryther (1962). The composition of the artificial seawater and the nutrients making up this growth medium are listed in the Appendix. A stock solution containing the trace metals was also obtained from A. Stiffey to facilitate composition of medium.

Continued survival of the culture required maintenance of a particular temperature and light environment. This was initially established in a small laboratory room, because the incubator malfunctioned. A temperature of 20 ± 3 °C was maintained with circulating air and timed lights established a 12-hour light, 12-hour dark, light cycle. The culture was transferred to provide familiarity with sterile transfer techniques and to increase the number of samples available. The cultures were then maintained in a stationary growth phase until another incubator was obtained. This incubator, a Model G27 from the New Brunswick Scientific Company, was loaned from the David Taylor Naval Ship Research and Development Center in Annapolis, Md. The cultures were transferred to this environment and maintained at 20 ± 2 °C. The same 12-hour light,

12-hour dark cycle was maintained using a 75-watt light bulb controlled by an AMF Model ADT 15-00 clock-operated switch. The light was placed about 55 cm from the cultures, producing a light intensity of 75-90 footcandles. The variations in intensity were caused by slight differences in the distances between the light and the various cultures. The cultures were rotated regularly in order to eliminate any minor effect this small change in light intensity might have. The beginning of the light phase, CT 0000, was set at 8 P.M. The middle of the dark phase, at which most experiments were conducted, occurred at 2 P.M. The usual day-night alternation of light was reversed to allow the experiments to be conducted during daytime hours.

A secondary transfer of the cultures was made to increase the number of samples available, and then the cultures were allowed to remain in a stationary growth phase until needed for the experiment.

Transfers of the cultures were made using sterile transfer techniques in order to eliminate or minimize the introduction of any contamination. All glassware was cleaned by washing with double-distilled water and with a sulfuric acid/potassium dichromate cleaning solution. It was then sterilized by autoclaving.

Once the "F/2" medium was composed, it was autoclaved at 15 psi (121 $^{\circ}$C) for twenty minutes, cooled, and stored in a cool, dark location. Sterile pipettes were flamed briefly with a Bunsen burner, then cooled and the transfer made. The

original culture was grown in a 1000 ml Erlenmeyer flask, while the transferred cultures were placed in 125 ml and 50 ml Erlenmeyer flasks, filled from one-third to one-half of their capacity.

B. Equipment

A schematic diagram of the experimental set up is shown in Figure 11. Pressure change was created in a stainless-steel pressure chamber (Figure 12), capable of withstanding high pressures well above those used in this experiment. It had a one ml container enclosed on top and bottom by one inch plexiglass windows secured by threaded bolts and sealed with O-rings and fitted washers. The bottom was secured by a solid bolt that prevented any light output, while the top bolt was cut-out to allow light detection. Pressure changes were induced and measured through ports on either side of the sample chamber.

Pressure changes were obtained through use of 85 psi laboratory compressed air. This pressure was regulated by means of a reduction valve attached to a reservoir (RV and RES in Figure 11). This assembly reduced the pressure to the desired valve and ensured that it would remain at a steady level, despite the frequent pulses required during the experiment. An electrically-activated three-way toggle valve controlled the actual admission of pressure to the chamber. The input air pressure was measured by a standard Clippard PSIG pressure gauge, with a range from 0-100 psi (PG in Figure 11). Pressure chamber pressure was measured by a 0-100 psi Data

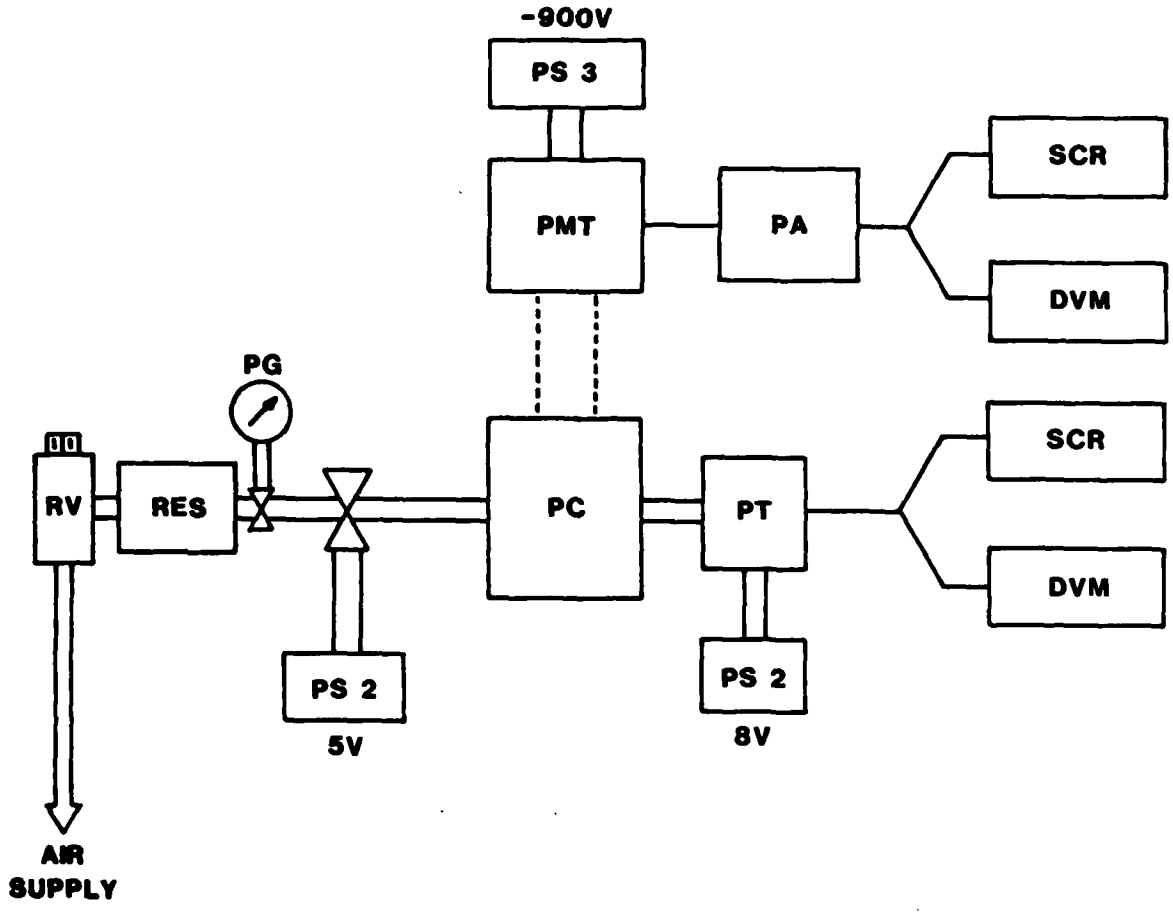


FIGURE 11: EXPERIMENTAL SETUP

KEY

RV - Reduction valve
RES - Reservoir
PC - Pressure chamber
PMT - Photomultiplier tube
PA - Pre-amplifier
PT - Pressure transducer
PS - Power supply
SCR - Strip chart recorder
DVM - Digital voltmeter
PG - Pressure gauge

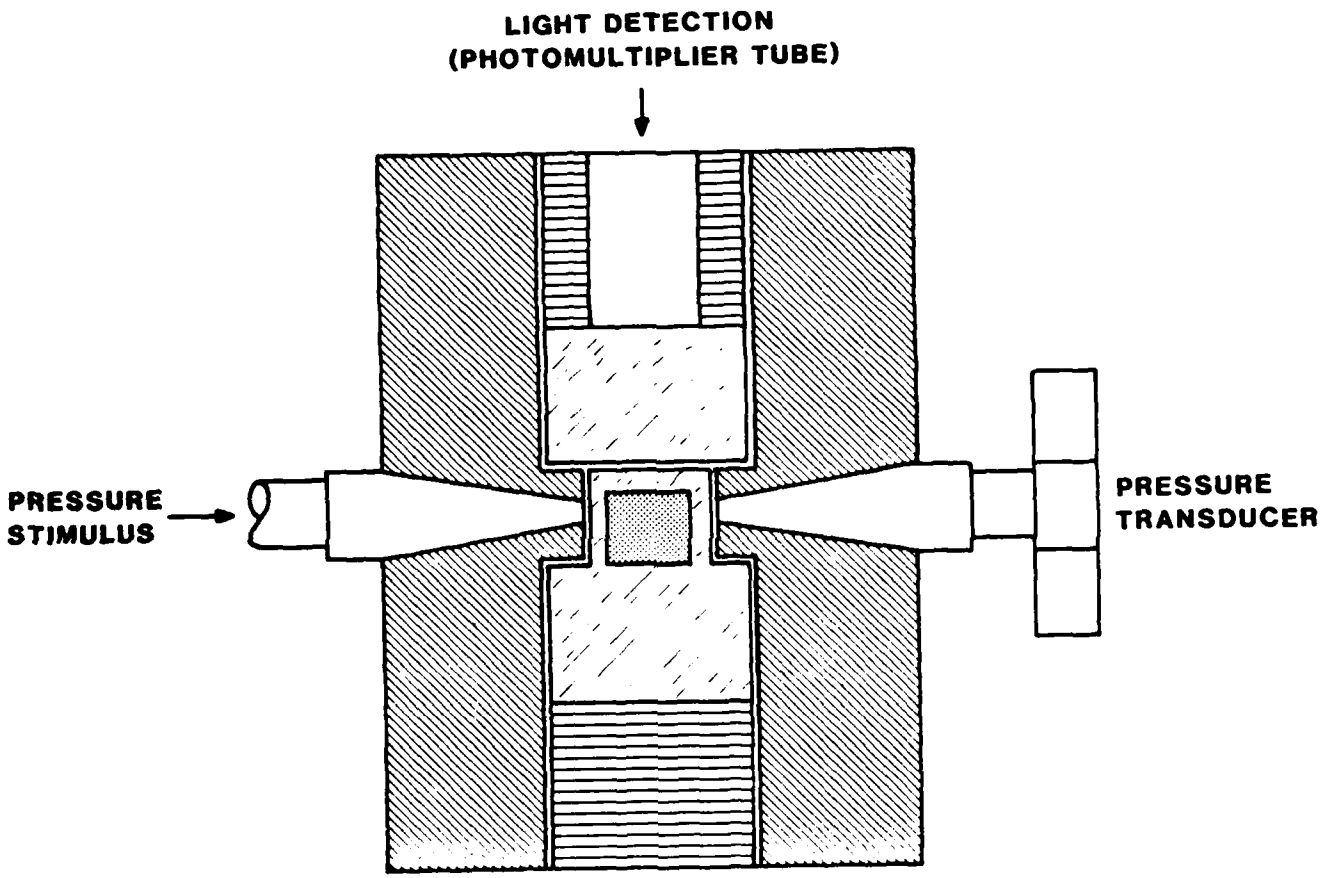


FIGURE 12: PRESSURE CHAMBER

Instruments, Model EA high gain, strain gage-type pressure transducer (PT in Figure 11).

A Textronix TM515 D.C. power supply (PS 1 in Figure 11) provided 8 V d.c. to the three-way toggle valve. A Hewlett Packard 6290A d.c. power supply (PS 2 in Figure 11) provided 5 V d.c. to the Data Instruments transducer.

The bioluminescent light output was detected by a 15 mm diameter, end window, RCA type 5819 S11 photomultiplier tube (PMT in Figure 11). It had a cathode sensitivity of 93 μAm/lm and required a constant, high voltage that was provided by a Hewlett Packard Harrison 6515A d.c. power supply (PS 3 in Figure 11). A negative supply voltage of -900 V was used for the experiments. The photomultiplier tube output required amplification to allow measurement. This was provided by an EG and G PARC Model 113 pre-amplifier (PA in Figure 11). The gain was varied between 200 and 1000 and output voltage was 1.0 V full scale.

Pressure signals, measured from the output of the transducer, and light signals, measured from the output of the pre-amp, were displayed digitally and recorded on a strip chart. The digital display was on two Textronix TM515 digital voltmeters (DVM in Figure 11). A simultaneous strip chart trace of pressure and light signals was made with a Gulton TR-722 Portable Two-Channel High Speed recorder (SCR in Figure 11).

IV. PROCEDURE

Prior to conducting the experiment, the cultures were transferred to put them in the active, logarithmic phase of growth. All handling of the samples throughout the experiment was gentle, with the aim of minimizing additional stimulation. The sample concentrations could not be determined, since the relatively large size of Pyrocystis lunula (>50 μm) precluded use of the available counting chamber.

The initial step in each test was to energize the equipment and ensure that the proper voltages were available. Before energizing the photomultiplier tube, it was necessary to close its aperture in order to prevent saturation by the room light. Next, the output voltages of the pre-amp and transducer were set and calibrated on the recorder. The pressure signal read 2.5 V full scale across the strip chart scale. Since 0.05 V corresponded to one psi, the strip chart scale ranged from 0 to 50 psi and each block represented a one psi change. Throughout the experiment, comparisons were made between the digital readout and the strip chart recording to ensure accuracy. After the voltages were set and the recorder calibrated, the initial pressure change was set and the toggle valve activated to compare the readings from the input pressure gauge and the transducer. A difference between the two indicated the presence of leaks in the system, which could then be corrected before the sample was tested.

Once the integrity of the system was assured, a one ml sample was gently pipetted into the test container and the

pressure chamber sealed. The photomultiplier was affixed to the open end of the pressure chamber and the junction was wrapped securely with black cloth to prevent interference from lights in the laboratory room. For the same reason, the lights in the room were restricted to the bare minimum required to operate the equipment. This care was required because of the high sensitivity of the photomultiplier tube. After the ambient light was minimized, the aperture of the photomultiplier tube was opened and monitoring of the sample began. The sample was allowed to rest for a minimum of 30 minutes to recover from any stimulation incidental to its transfer. During this period the sample was monitored constantly to determine if any spontaneous flashing or accidental stimulations occurred.

Once sufficient time had elapsed, the samples were subjected to a positive, then a negative pressure change, by activating the toggle valve. This was accomplished via a push button, allowing fast, precise control of the pressure change. The first factor determined was the pressure threshold required for light emission. To do this the pressure change was increased by increments of approximately .5 psi after a minimum of three pulses at a lower pressure elicited no response. The pressure was increased until the first light response was detected, thereby identifying the threshold. The pulses were of about a two-second duration with a two-second rest between pulses for a total period of four seconds. A portion of the strip chart record is shown in Figure 13, with pressure changes

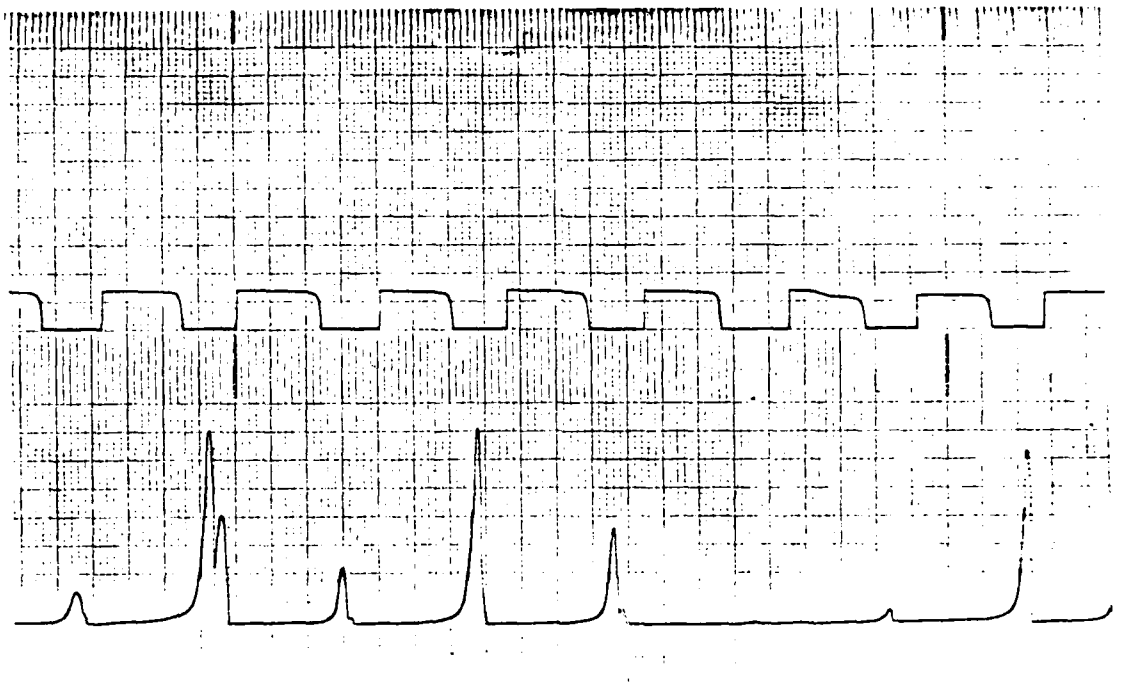


FIGURE 13:
STRIP CHART RECORDING OF PRESSURE PULSES AND BIOLUMINESCENT RESPONSE

UPPER TRACE: PRESSURE PULSES OF 7.0 PSI MAGNITUDE AND 2 SECOND DURATION (4 SECOND PERIOD)

LOWER TRACE: BIOLUMINESCENT RESPONSE IN ARBITRARY UNITS

on the upper trace and light output on the bottom trace. As shown, the trace reads from right to left. After completion of the test, the sample was removed from the chamber to a separate flask in order to prevent interference with the untested organisms.

Evaluation of the effect of varying the rate of pressure change was accomplished by changing the rest period after each pulse as well as using a one-second pulse, one-second rest cycle. The rest period was increased to ten and then twenty seconds. Preliminary tests showed that the length of the pulse was not significant, so it was not studied in detail. Most measurements were made at or near the middle of the scotophase, but some observations were made near its beginning and end in order to evaluate the effect of the circadian rhythm on sensitivity to stimulation.

V. RESULTS

Figure 13 is a typical bioluminescent response to a series of pressure changes above the threshold level. Pressure decreases are much more effective in stimulating bioluminescence than pressure increases. As a result of this, responses from pressure increases are not included in the data analysis. Figure 14 is a summary of the results of 47 experiments made with pulses of a two-second duration followed by a two-second rest. The mean decrease threshold was 5.10 $\pm$ 1.70 psi and 83% of the samples were between 3.0 and 7.5 psi.

To evaluate the effect of rate change on sensitivity to stimulation, experiments were conducted using three different cycles, in addition to the original two-second pulse, two-second rest cycle. Preliminary data indicated that pulse length was not a significant factor, so it was not subjected to further analysis. Seven runs on a one-second pulse, one-second rest, cycle yielded a stimulation threshold of 4.76 $\pm$ .99 psi. Eight runs on a two-second pulse, ten-second rest cycle yielded a threshold of 5.51 $\pm$ 1.42 psi. Seven runs on a two-second pulse, twenty-second rest cycle yielded a threshold of 5.41 $\pm$ 1.33 psi. In Figure 15 are comparisons of the threshold levels for the different rates of pressure change. It was also observed that samples stimulated at faster rates emitted flashes of relatively larger amplitude and tended to fatigue sooner than those pulsed at a slower rate.

Experiments were conducted at times other than the middle of the scotophase to determine the effect of the circadian

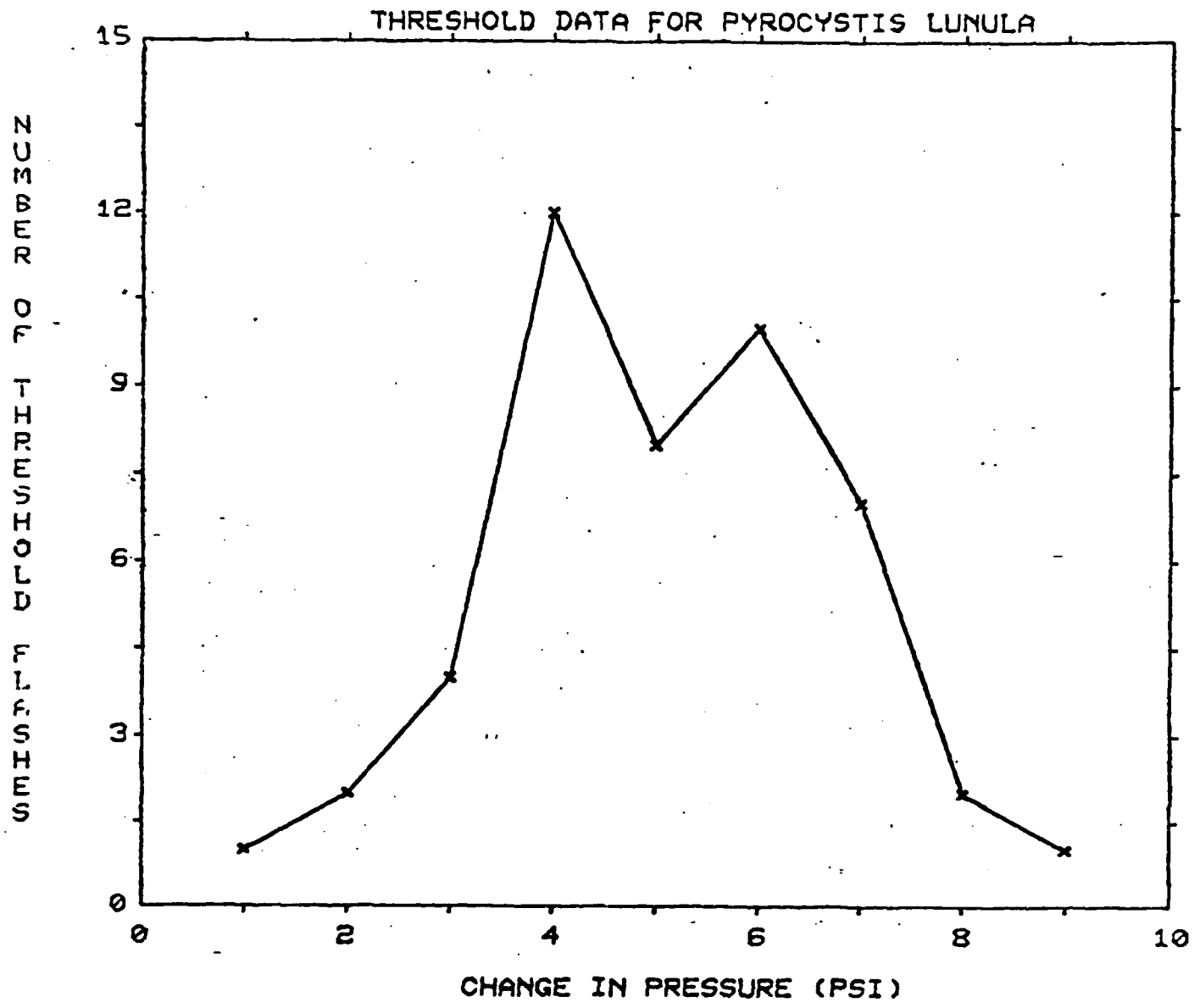


FIGURE 14: THRESHOLD DATA FOR PYROCYSTIS LUNULA

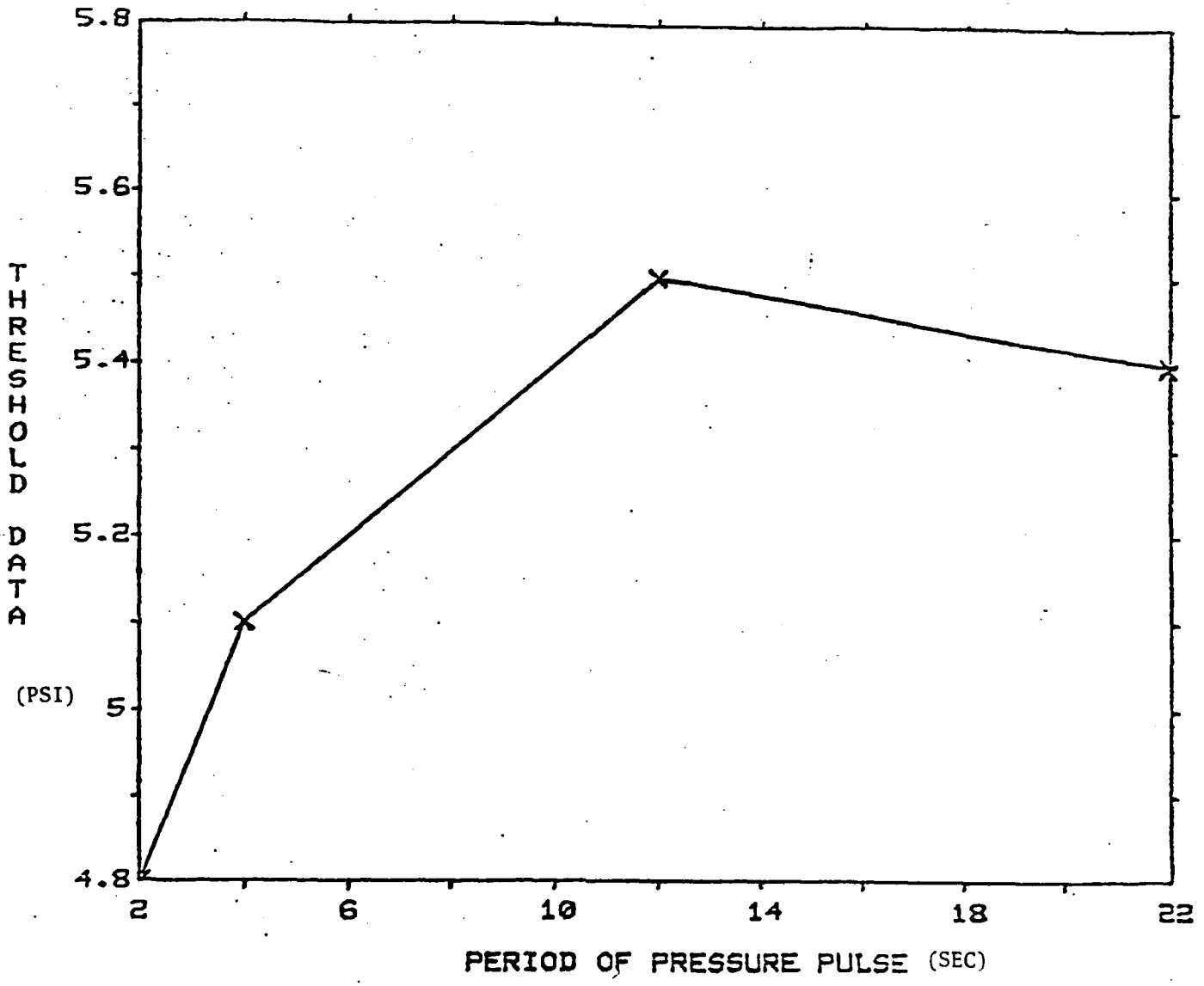


FIGURE 15: VARIATION OF THRESHOLD DATA WITH RATE OF CHANGE

rhythm. No significant differences in threshold levels were obtained for most times within the scotophase with the exception of the 30 to 45 minutes at the beginning and end of the scotophase. During this time a considerably greater magnitude of pressure change was required to stimulate luminescence. During the photophase it was no longer possible to stimulate a detectable light emission.

VI. DISCUSSION AND APPLICATION OF RESULTS

The selection of Pyrocystis lunula for this experiment was based on its ready availability in culture and its wide oceanic distribution. Further advantages of its use were revealed during the experiment, as it demonstrated a relatively hardy nature and excellent light response characteristics. The light emitted by Pyrocystis was characterized by bright flashes and a lack of spontaneous flashing that made it easy to work with in light stimulation studies. This experiment only tested for the occurrence of a luminescent response, and did not seek to measure the actual light output. For this reason, it was not essential to determine the precise concentrations of the cultures. The samples were stirred gently before they were placed in the chamber to ensure uniform concentration. This also served to minimize the difference between the densities of different samples, effectively eliminating it as a variable.

The definite response of Pyrocystis lunula cultures to slight, controlled pressure changes demonstrates the effectiveness of these changes as stimuli. The data did not demonstrate as distinct a threshold as expected, but there was a definite concentration between 3.0 and 7.5 psi. A larger number of samples would have provided greater statistical certainty.

Pressure decreases were found to be much more effective than increases, in terms of both the amplitude and frequency of the emitted light. The response to pressure increases was limited to relatively small peaks of light output caused by fairly large (> 10 psi) pressure changes. Initially, there

appeared to be a large response to pressure increases, but this was traced to a pressure leak that caused minor vibrations when the system was pressurized. After this leak was sealed, the effect of pressure increases became negligible and was disregarded in further analyses. These data confirm previous findings by Donaldson (1982). The cause of this striking difference was attributed at first to decompression of gas bubbles with lowered pressure, but the small magnitudes of pressure changes involved virtually eliminated that as a possibility. It has been suggested that the organism's response is determined by the sign of the differential change in pressure (Donaldson, 1982). The strong dependence on a pressure decrease as a stimulus was also reflected in the relative insignificance of pulse length as a stimulus factor. It was important only as it influenced the length of time between pressure decreases. It is for this reason that the threshold pressure data are presented in psi rather than psi per second.

The rate of pressure change affected the stimulation of light in several ways. A faster rate appeared to reduce the amount of pressure needed to initially stimulate light emission. Further data are required to positively establish this relationship. The relationship between the rate of stimulation, the amount of light output per flash, and the time to fatigue has undergone considerable investigation. Widder and Case (1981) found that organisms subjected to repetitive mechanical stimulation took more time to fatigue at low

stimulus frequencies. Though more flashes were generated, the amplitude of each is less than those of higher frequencies, so the total light output remained constant. Though this experiment was not concerned with determining the amount of light emitted, observations of the light emitted by samples stimulated to fatigue roughly agreed with the constant total stimulable light hypothesis.

Several other interesting features of bioluminescent emissions were observed during the course of this experiment. The first flash of a previously unstimulated organism had a markedly sharper rise time and greater amplitude than the following flashes. The most likely cause of this was a synchronous response by the majority of the organisms in the sample. Differences in decay and recovery times prevented such synchronous action in the subsequent flashes. A delay of 0.2 to 0.4 seconds was observed between stimulus and response in the organism. This was a function of both a delay in transmitting the mechanical stimulus across the cell membrane as well as a delay in transmitting the pressure change through the system. Another feature noted as a characteristic of many flashes was a double peak, shown in Figure 13. The most likely cause of this phenomenon was an uneven response by the organisms within the sample. As the pressure change was induced, the organisms near the surface of the sample may have responded first, followed by those further away. The distances involved were miniscule, but they may have been sufficient to generate the double peak. Another interesting observation

concerns the alternation of high and then low amplitude responses to pressure decreases. This is visible in Figure 13 and was particularly evident at higher rates of change. It appeared to indicate a need for a certain amount of rest before the next response. Further investigation is necessary to determine the true cause.

The effect of the circadian rhythm on light emission within either phase was not observed to be a significant factor. This is in agreement with the predomination of the light/dark cycle in creating a square wave response that remains nearly constant throughout the scotophase. The significant decrease in sensitivity to stimulation near the beginning and end of the scotophase and the nearly complete loss during the photophase also agrees with previous results.

The threshold data may be applied to a streamline flow model, in order to determine the location of bioluminescence around a moving hull (Chapman, 1982). This model was used by Donaldson (1982) in a similar analysis of flow along a rounded, submerged cylinder that may be treated as a simplified submarine. Chapman's calculations depend on Laplace's equations for potential flow. Scaling was used to increase the model speed from the designed model speed of 7 m/s to a projected hull speed of 10 m/s (20 kts). A complete description of the model is available in Chapman (1982). Basically, the velocity field induced by a moving body will cause the scalar properties of the field, such as pressure, to move with the fluid. If the velocity field is assumed to be represented

by horizontal streamlines, conservation of energy along those streamlines creates a balance between the pressure force and velocity distribution. Since the velocity distribution is known, the pressure field may be calculated (Donaldson, 1982). In developing the model and applying the data certain assumptions were made. These include an incompressible fluid, horizontal streamlines, and irrotational flow along the streamline. Dinoflagellate organisms are regarded as point receivers of pressure field forces. Bioluminescence will occur whenever the induced pressure field exceeds the 5.10 psi/s threshold level. For the reasons discussed earlier concerning pulse length, the pressure change was applied to the model as a rate change of an arbitrary one-second duration. Figure 16 (Donaldson, 1982) has an example of the coordinate system to which the model is referenced, while the streamline flow is shown in Figure 17 (Donaldson, 1982). The pressure distribution field for a 20 kt velocity is listed in Table 2. The location at which bioluminescence occurs at a 20 kt velocity is shown by the heavy line in Figure 18. It takes place out to an average distance of 2.2 m from the hull.

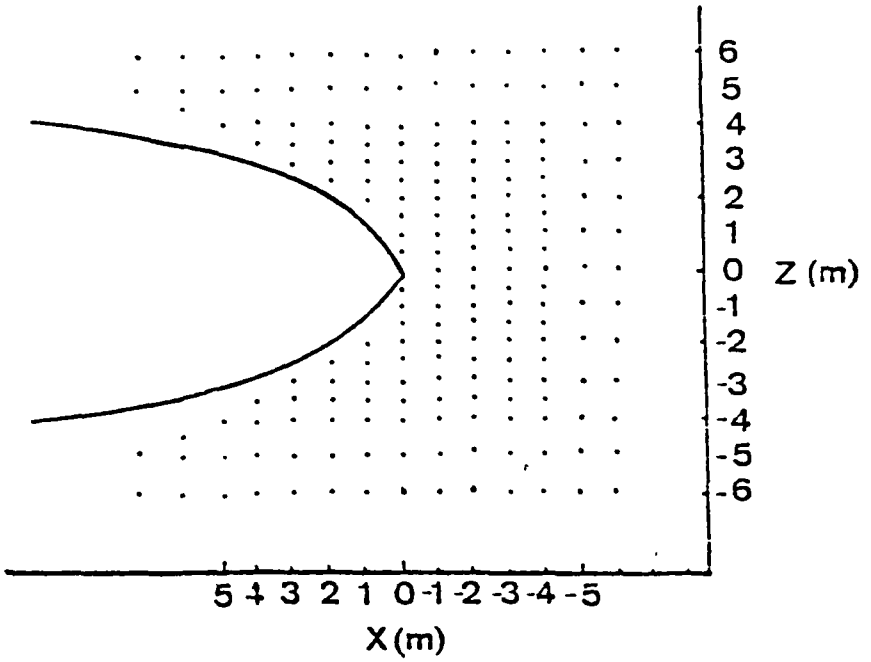


FIGURE 16: MODELLED OBJECT AND COORDINATE SYSTEM

(from Donaldson, 1982)

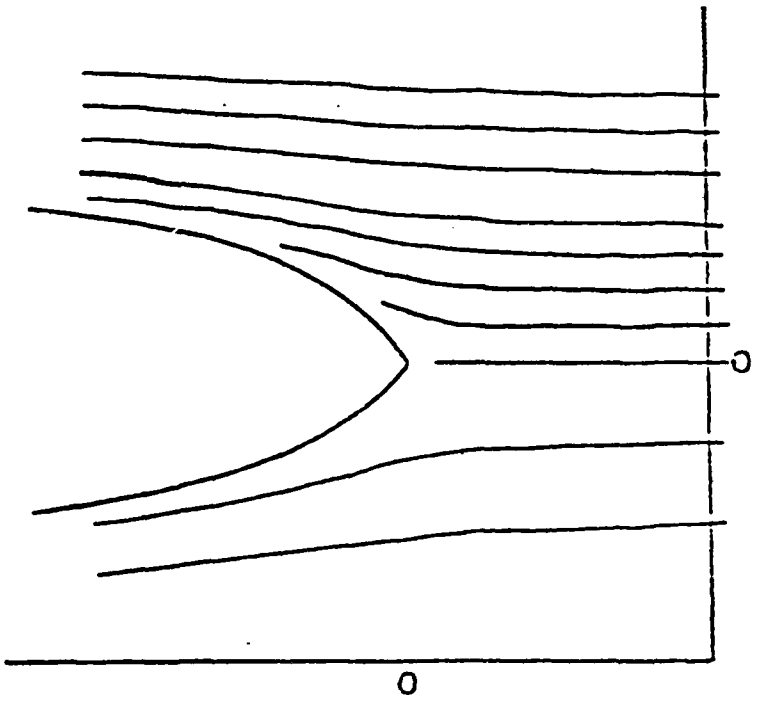


FIGURE 17: CALCULATED STREAMLINE FLOW

(from Donaldson, 1982)

TABLE 2

PRESSURE RATE OF CHANGE VALUES [PSI/s]
FOR 20 KT VELOCITY. (FROM CHAPMAN, 1982.)

Z (m) \ X (m)	-3.5	-2.5	-1.5	-.5	.5	1.5	2.5	3.5
-4.0	1.13	1.19	1.07	.34	-.63	-2.14	-3.33	-3.77
-3.5	1.38	1.59	1.6	1.05	-.54	-3.02	-4.86	
-3.0	1.63	1.97	2.10	1.53	-.55	-4.0	-6.5	
-2.5	1.93	2.5	3.05	2.83	.044			
-2.0	2.22	3.02	3.92	3.9	.047			
-1.5	2.49	3.59	5.24	6.82				
-1.0	2.74	4.13	6.4	8.8				
- .5	2.85	4.39	7.17	11.3				
0	2.96	4.64	7.82	12.22				
.5	2.86	4.40	7.17	11.28				
1.0	2.75	4.14	6.415	8.84				
1.5	2.5	3.61	5.26	6.87				
2.0	2.24	3.05	3.95	3.96	.162			
2.5	1.95	2.54	3.09	2.9	.065			
3.0	1.65	2.0	2.15	1.57	-.446	-3.84	-6.26	
3.5	1.4	1.62	1.65	1.11	-.445	-2.88	-4.66	
4.0	1.15	1.23	1.12	.579	-.531	-2.01	-3.16	-3.34

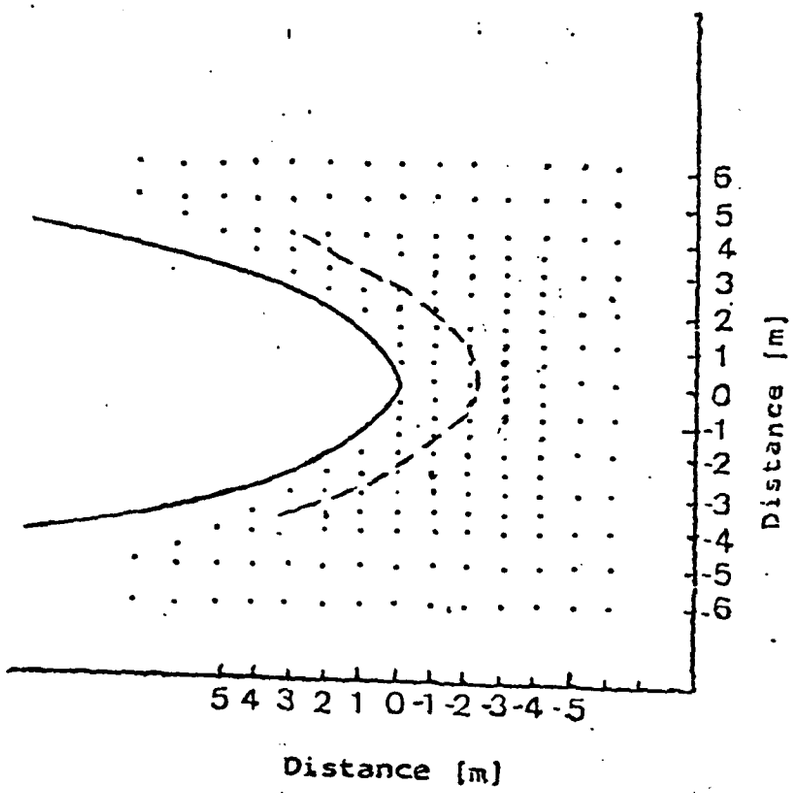


FIGURE 18: BIOLUMINESCENCE CONTOUR FOR 20 KT FLOW

(adapted from Donaldson, 1982)

VII. CONCLUSIONS AND RECOMMENDATIONS

The results of this experiment confirm the effectiveness of controlled pressure changes of relatively small magnitude in inducing bioluminescence in Pyrocystis lunula. Stimulation is dependent on the sign of the pressure change, and pressure decreases were found to be much more effective. A mean level of 5.10 ± 1.70 psi was determined as the threshold stimulus for samples tested on a two-second pulse, two-second rest cycle.

The analysis of rate of pressure change, while not statistically significant, did indicate that faster rates of stimulation induced luminescence with a pressure change of lower magnitude. The threshold ranged from $4.76 \pm .99$ psi for a fast rate of change to 5.51 ± 1.42 psi for a slower rate. In evaluating the rate of change, the most important factor was the interval between pressure drops. The length of the pulse did not have a noticeable effect on the stimulation level. Analysis of other factors, including the circadian rhythm and the kinetic characteristics of the emitted light, demonstrated agreement with previous findings.

The threshold data was applied to a pressure field around a submerged cylinder modeled at a speed of 20 kts. Bioluminescence was predicted to occur from the hull to a distance of 2.2 m from the tip of the cylinder.

The use of small-magnitude, controlled pressure changes to stimulate bioluminescence is a relatively new approach and requires considerably more research. It is a valuable tool for gaining further understanding of the bioluminescent process.

Further analysis of the interaction of rate and magnitude changes on stimulated luminescence is necessary, and the effect on both the frequency and amount of light output should be evaluated. The effectiveness of pressure stimulation should be tested with other bioluminescent organisms to see if it has widespread applications.

In addition to laboratory studies, a concerted effort is needed to evaluate the distribution of bioluminescence in the ocean. Current development of an airborne or satellite-based sensing system would greatly facilitate such a study. A greater understanding of bioluminescence is required in order to accurately predict its potential effects on detection systems. The Navy cannot afford to ignore an environmental factor of such significance as bioluminescence.

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APPENDIX

Growth Medium for *Pyrocystis lunula*

F/2 Medium from Guillard and Ryther, 1962

$NaNO_3$			150 mg
NaH_2PO_4 H_2O			10 mg
Thiamine HCl			200 mg
Biotin			1 mcg
B_{12}			1 mcg
Fe^{2} Sequestrene	5 mg	Fe	.65 mg
$CuSO_4$ $5H_2O$		Cu	.0025 mg
$ZnSO_4$ $7H_2O$		Zn	.005 mg
Co Cl_2 $6H_2O$		Co	.0025 mg
$MnCl_2$ $4H_2O$		Mn	.050 mg
Na_2MoO_4 $2H_2O$		Mo	.0025 mg
THAM			5.0 grm

Lyman Fleming artificial sea water 1000 ml

Lyman Fleming Artificial Sea Water (Modified)

NaCl	23.477 grm
$MgCl_2$	4.981 grm
Na_2SO_4	3.917 grm
$CaCl_2$	1.102 grm
KCl	0.664 grm
$NaHCO_3$	1.44 grm
KBr	0.096 grm
H_3BO_3	0.026 grm
$SrCl_2$	0.024 grm
NaF	0.003 grm

Distilled deionized water to 1000 ml